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From: Romeo, David
Sent: Tuesday, November 10, 1998 4:09 PM
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Subject: 08/981,559 Brakebusch C; Nophar Y; Kemper O; Engelmann H; Wallach D

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Borrower's Name ... David Romeo
    Org or A.U. ... 1646, Mailbox, 10E18
          Phone ... 305-4050
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Date of Request ... 10 November 98
 Date Needed by ... 17 Nov 98
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AU   Brakebusch C; Nophar Y; Kemper O; Engelmann H; Wallach D
TI   Cytoplasmic truncation of the p55 tumour necrosis factor (
       ***TNF***  ) receptor abolishes signalling, but not induced shedding
     of the receptor.
SO   EMBO JOURNAL, (1992 Mar) 11 (3) 943-50.
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Cytoplasmic truncation of th p55 tumour necrosis factor (TNF) receptor abolishes signaiiing, but not induced shedding of the receptor

Cord Brakebusch, Yaron N phar, Oliver Kemper, Hartmut Engelmann and David Wallach

The Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot, Israel 76100

Communicated by S.Fuchs

The mechanistic relationship between the signalling for the TNF effects by the human p55 TNF receptor (hu-p55-TNF-R) and the formation of a soluble form of the receptor, which is inhibitory to these effects, was explored by examining the function of C-terminally truncated mutants of the receptor, expressed in rodent cells. The 'wild-type' receptor signalled for a cytocidal effect when cross-linked with specific antibodies and exhibited spontaneous shedding. Shedding of the receptor was not affected by TNF but was markedly enhanced by 4β-phorbol-12-myristate-13-acetate (PMA). Receptor mutants with 53%, 83% and 96% C-terminal deletions could not signal for the cytocidal effect. Furthermore, they were found to associate with the endogenous rodent receptors, interfering with their signalling. Yet even the deletion of 96% of the intracellular domain did not abolish shedding of the receptor in response to PMA. These findings suggest that signalling and shedding of the p55 TNF-R are mechanistically distinct.

Key words: receptor mutants/shedding/soluble receptors/structure–function relationship/TNF receptor hu p55

Introduction

Tumour necrosis factor (TNF), a pro-inflammatory cytokine produced primarily by mononuclear phagocytes, contributes to the defence of the host against pathogens as well as to various detrimental manifestations of inflammation through a variety of different effects on cell function (Old, 1990; Beutler and Cerami, 1989). These effects are initiated by the binding of TNF to specific, high affinity receptors, which are expressed on the surface of most kinds of cells (Baglioni *et al.*, 1985; Aggarwal *et al.*, 1985; Beutler *et al.*, 1985; Kull *et al.*, 1985; Tsujimoto *et al.*, 1985; Israel *et al.*, 1986). The receptors provide the intracellular signals for cell response to TNF (Engelmann *et al.*, 1990a). Two molecular species of the TNF receptors (TNF-Rs), expressed differentially in different types of cells, have been identified (Engelmann *et al.*, 1990b; Brockhaus *et al.*, 1990). Both exist also in soluble forms (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989), which are derived proteolytically from the cell surface receptors (Nophar *et al.*, 1990; Porteu and Nathan, 1990; Porteu *et al.*, 1991). The soluble forms of the TNF receptors specifically bind TNF, and thus, by competing for its binding t the cell surface TNF-Rs, may function as inhibitors of TNF activity (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989). Their formation *in vitro* is enhanced by certain stimulatory agents, including the tumour promoting phorbol diester 4β-phorbol-12-myristate-13-acetate (PMA), and the chemotactic peptide formyl-methionine-leucine-phenylalanine (in granulocytes) (Porteu and Nathan, 1990; Lantz *et al.*, 1990; Kohno *et al.*, 1990). Their serum concentrations increase dramatically in various diseases (Aderka *et al.*, 1991 and unpublished data), quite probably as a consequence of cell exposure *in vivo* to such stimulants.

Little is known of the mechanisms of signalling, protein cleavage and membrane trafficking which take part in TNF-R function, or of the extent to which these different activities are interlinked. The recent cloning of the cDNAs for the two TNF receptors (Heller *et al.*, 1990; Loetscher *et al.*, 1990; Nophar *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990) has provided access to structure–function analysis of the mechanisms of action and modulation of these receptors. In the present study, we explored the mechanistic relationship between the signalling activity and the inducible shedding of the p55 TNF-R by examining the effect of cytoplasmic truncation of the receptor on these activities.

Results

Signalling for the cytocidal effect of TNF in rodent cells by transfected human p55 TNF receptors

In order to identify the structural elements in the p55 TNF-R specifically involved in the mediation of distinct activities, mutated forms of this receptor were created and expressed in cultured cells. Since most cell lines express TNF receptors, it was necessary to find a way of distinguishing between the activities of the receptors encoded by the expression constructs and those endogenous to the cells. Cross-linking the p55 receptors by antibodies recognizing their extracellular domain triggers their signalling activity (Espevik *et al.*, 1990; Engelmann *et al.*, 1990a). We therefore expressed the human p55 receptor in rodent cells and used specific antibodies with the aim of triggering these receptors selectively, without affecting the receptors endogenous to the rodent cells.

After deletion of large parts of the 3′ and 5′ non-coding sequences, the cDNA for the human p55 TNF receptor (hu-p55-TNF-R) was introduced into the pMPSVEH expression vector, under the control of the myeloproliferative sarcoma virus promoter (Artelt *et al.*, 1988) and co-transfected with an expression vector encoding the neomycin resistance gene into cells of the murine A9, L929 and NIH 3T3 lines and of the hamster BHK line. Cells constitutively expressing the transfected vectors were selected by growth in the presence of G418. As controls, cells transfected with the neomycin resistance vector alone were selected by the same procedure. A large proportion of the clones co-transfected with the vector containing the wild-type (or mutants, see below)

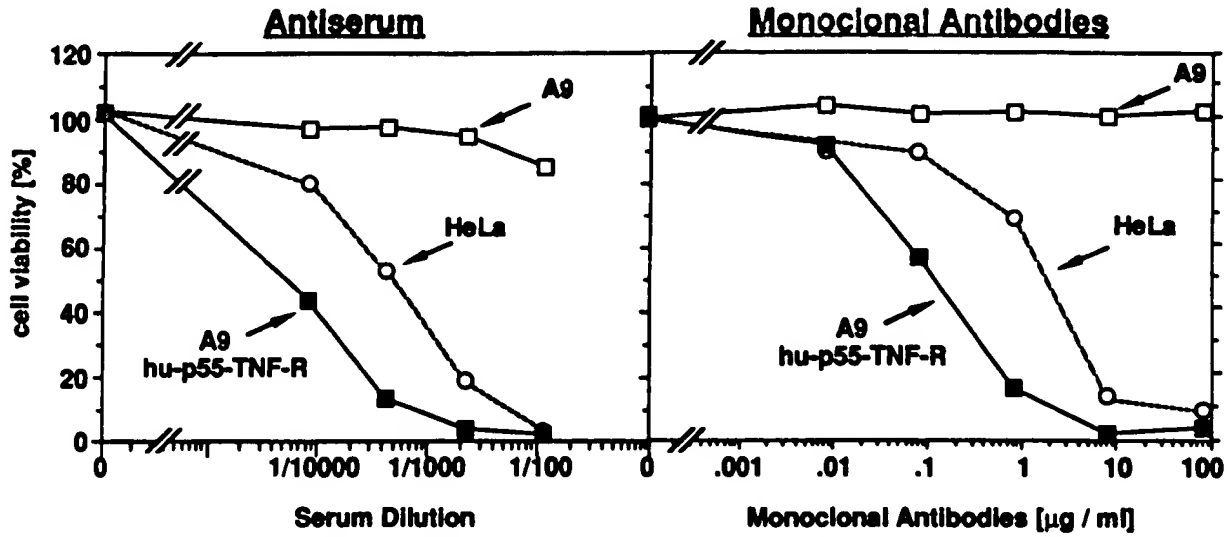

Fig. 1. Cytocidal effects of rabbit antiserum against the hu-p55-TNF-R (left panel), and murine monoclonal antibodies against the receptor (right panel) in HeLa cells (○), A9 cells (□) and A9 cells expressing the wild type hu-p55-TNF-R (■). The antiserum, or the monoclonal antibodies (numbers 18 and 20, Engelmann *et al.*, 1990b), were applied simultaneously with CHI (50 µg/ml in the A9 cells and 25 µg/ml in the HeLa cells). The two monoclonal antibodies were applied in equal amounts, to the concentration specified in the figure.

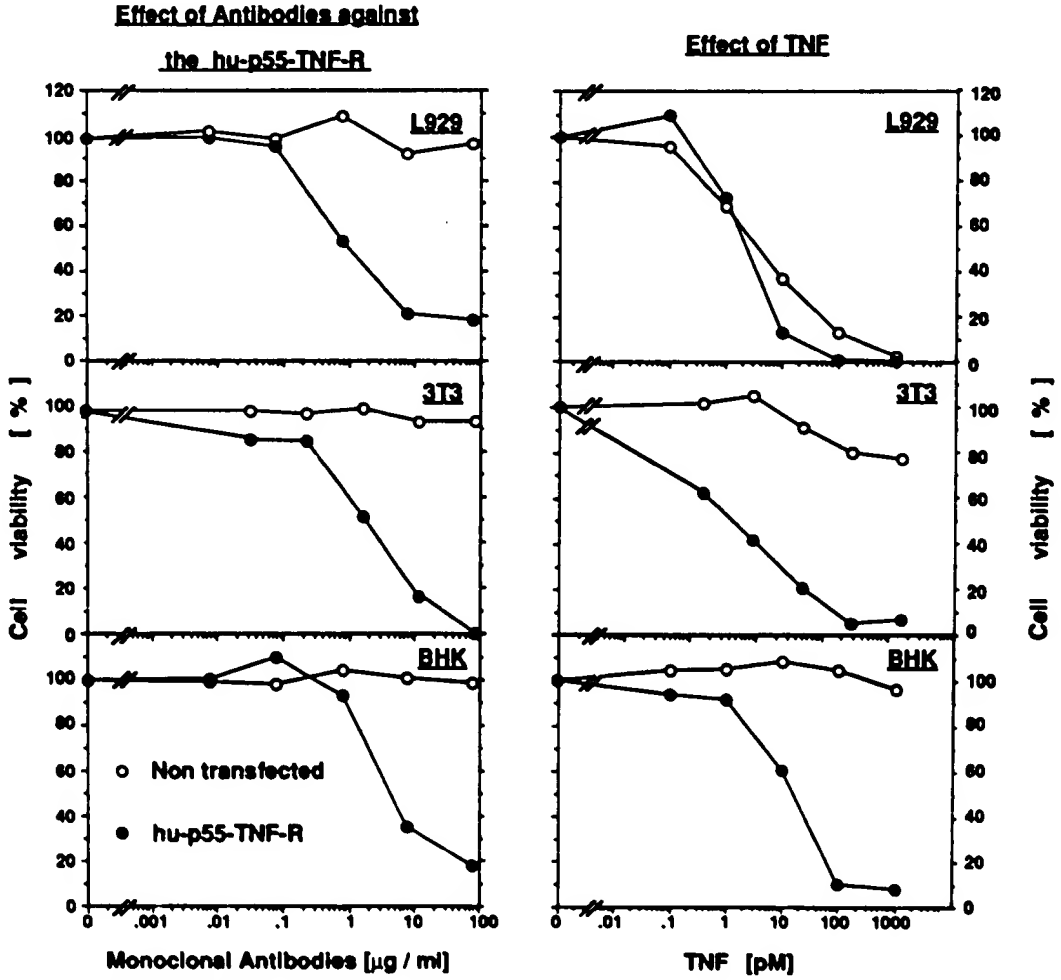

Fig. 2. Response of the L929, NIH 3T3 and BHK cells (○) and of those same cells when expressing the wild type hu-p55-TNF-R (●), to the cytocidal effect of monoclonal antibodies against the hu-p55-TNF-R (left panels) and of TNF (right panels). The assay was performed as in Figure 1.

hu-p55-TNF-R cDNAs (>50% of the 25–50 clones picked up from each transfected culture) showed a significant increase in TNF binding, up to 10-fold that observed in the non-transfected cells, indicating effective expression of the cDNAs. However, TNF binding by cells transfected only with the neomycin resistance vector was indistinguishable from the binding observed in the non-transfected cells. Cells showing highest TNF binding were chosen for further analysis, testing at least three and in most cases more than five clones for each DNA construct.

Effective cross linking of the p55 TNF receptors in order to trigger their signalling activity can be achieved with polyclonal antisera or by simultaneous application of two different monoclonal antibodies each recognizing different epitopes in the extracellular domain of the receptor (Engelmann *et al.*, 1990a). While in the human HeLa cells, antibodies against the TNF receptor induced a pronounced TNF-like cytocidal effect (Figure 1), in non-transfected murine A9 cells, which are equally sensitive to the cytocidal effect of TNF, rabbit antiserum against the hu-p55-TNF-R had ony a slight effect (Figure 1, left panel), and mouse monoclonal antibodies had no effect at all (Figure 1, right panel). The antibodies had no effect either in control-transfected cells expressing only the neomycin resistance gene (not shown). In contrast, both the polyclonal and monoclonal antibodies had a strong cytocidal effect on A9 cells in which the hu-p55-TNF-R was expressed, comparable to their effect on the HeLa cells (Figure 1). Similarly, the monoclonal antibodies against the hu-p55-TNF-R were markedly cytocidal to L929, NIH 3T3 and BHK cells expressing the hu-p55-TNF-R, though no such effect could be observed in the non-transfected cells, nor in cells expressing only the neomycin resistance gene (Figure 2, left panels, and data not shown). Comparison of the response to the antibodies in different clones of the transfected cells (3–20 clones of each transfected line) revealed a rough proportionality between the extent of response and the extent of expression of the transfected receptors, as reflected in TNF binding to the cells. Thus, in a given experiment, applying the rabbit antiserum at a dilution of 1:2500 to cells of seven different transfected A9 clones, exhibiting increased TNF binding of 0.5-fold, 1.3-fold, 3.3-fold, 5.6-fold, 7-fold and 12-fold (above the binding of 510 CPM measured in the control cell cultures, expressing only the neomycin resistance gene), resulted in death of 7, 25, 43, 52, 87 and 92% of the cells, respectively. These findings indicate that the human receptor provides signal(s) which are sufficient to elicit an effective response in rodent cells.

Another reflection of the functionality of the human receptors in rodent cells was an increased response of the transfected cells t TNF itself. This increase was barely detectable in the A9 and L929 cells, which are highly sensitive to the cytocidal effect of TNF, but was highly significant in the NIH 3T3 cells, and in the BHK cells, which normally respond rather poorly to TNF cytotoxicity (Figure 2, right panels and data not shown). Expression of the neomycin resistance gene alone had no effect on the response of the cells to TNF (not shown).

Loss of signalling activity in truncated forms of the human p55 TNF receptor and evidence for interaction of these truncated forms with the endogenous, full-length mouse receptors

Since the functional regions in the intracellular domain of the p55 TNF are not known, a choice of mutant receptors whose intracellular domains were truncated by ~53%, 83% and 96% (mutants Δ:310-426, Δ:244-426 and Δ:215-426, respectively, in Figure 3), was made arbitrarily. cDNAs encoding the truncated receptors, generated by introducing stop codons into the coding region, and fused with the myeloproliferative sarcoma virus promoter, were expressed in the murine A9 cells (see Materials and methods).

Cells expressing either one of the receptor mutants showed increased TNF binding, comparable in extent to that in cells expressing the wild-type receptor. Also, the affinities of the expressed mutant and wild-type receptors to TNF were similar. Thus, analysis of TNF binding to cells of A9 clones expressing the wild-type hu-p55-TNF-R receptor, or the Δ:310-426, the Δ:244-426 or the Δ:215-426 receptor mutants, showed that the number of receptor sites per cell in these clones were 8070, 10 500, 3600 and 14 900, respectively, and their affinity for TNF were 2.8 ± 0.21 × 10^{-10} M, 5.3 ± 0.61 × 10^{-10} M, 7.2 ± 2.36 × 10^{-10} M and 6.8 ± 0.92 × 10^{-10} M respectively. The mutant receptors also facilitated TNF uptake and degradation in the A9 cells, although at a lower rate than that of the full-length receptors, proportionally to the degree of receptor truncation (data not shown).

To obtain information on the signalling activity of the truncated forms of the hu-p55-TNF-R, we determined their

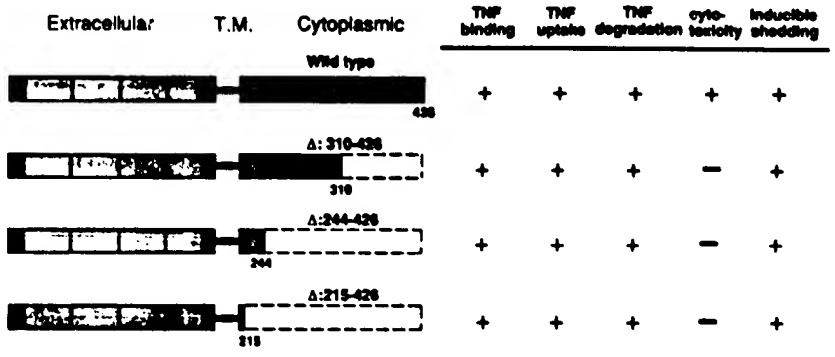

Fig. 3. Structure of the wild-type human p55 TNF receptor and its mutants and a summary of their activities. Icons depicting the full length and truncated forms of the human p55 TNF-R. A summary of the data on the activities of the wild-type and mutant receptors is presented in the right hand columns. A plus sign indicates activity retained, and minus indicates activity lost.

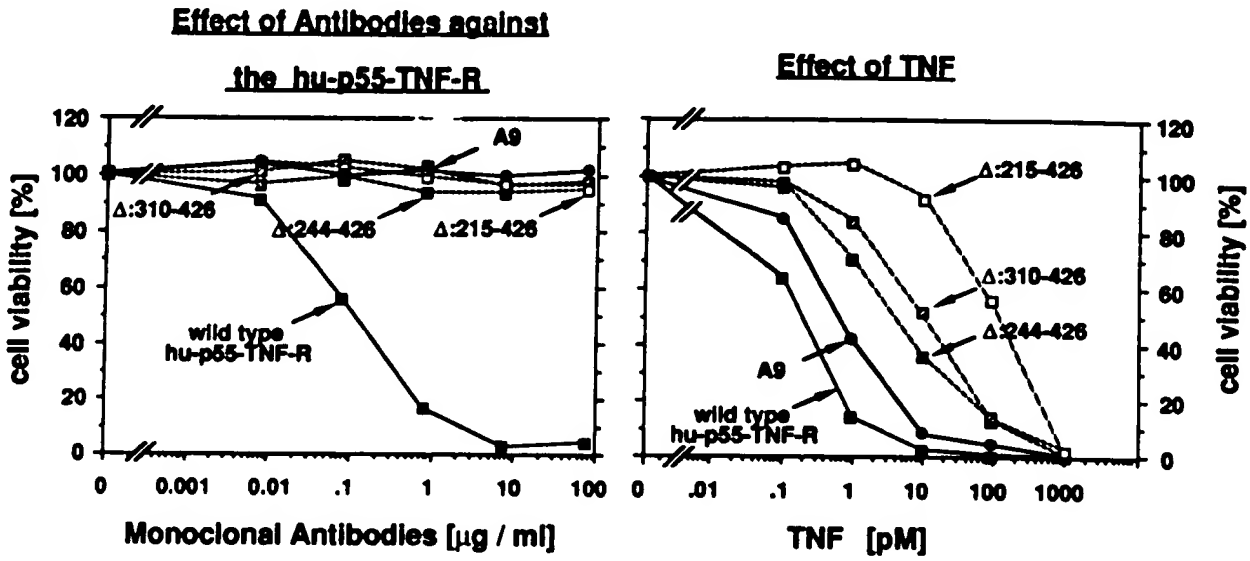

Fig. 4. Cytocidal effects of monoclonal antibodies against the hu-p55-TNF-R (left) and TNF (right), in A9 cells (●), in A9 cells expressing the wild-type hu-p55-TNF-R (■) and in A9 cells expressing the cytoplasmic deletion mutants of the hu-p55-TNF-R (Δ:310-426, ■; Δ:244-426, ▨; and Δ215-426, □). The assays were performed as described for Figure 1.

ability to elicit a cytocidal effect upon cross-linking with anti-receptor antibodies. While having a pronounced cytotocidal effect in the A9 cells which express the full-length hu-p55-TNF-R, the antibodies had no effect at all on cells expressing any of the three truncated forms of the receptor, suggesting that these truncated forms are not functional (Figure 4, left panel). Furthermore, testing the effect of TNF itself we found that, in contrast to the full-length hu-p55-TNF-R, whose expression increases the sensitivity of cells to TNF, the truncated forms of the receptors decreased the sensitivity to the cytocidal effect of TNF. This decrease in sensitivity was observed in multiple clones of A9 cells expressing any one of the truncated forms of the receptors (Figure 4, right panel) at a degree which seemed roughly proportional to the extent of receptor expression (not shown). To confirm that the lowered response indeed reflects an inhibitory effect of the truncated receptors on the endogenous rodent receptors, the effect of TNF was examined also in the presence of antibodies to the hu-p55-TNF-R, known to inhibit TNF binding to it (see Figure 2 in Engelmann *et al.*, 1990a). As shown in Figure 5, when treated with these antibodies, cells which express the truncated receptors recovered their sensitivity to the cytocidal effect of TNF.

The inhibitory effect of the truncated human receptors on the response of the A9 cells to TNF indicated that they interact with the endogenous murine receptors, interfering with their function. More direct evidence for this interaction was obtained in an SDS–PAGE analysis of the size of the truncated receptors, immunoprecipitated using antibodies against the hu-p55-TNF-R from extracts of transfected cells whose receptors were tagged by cross-linking to radiolabelled TNF (Figure 6). Although the antibodies used for the immunoprecipitation recognized specifically the receptors of human origin and thus did not precipitate TNF receptors from extracts of non-transfected A9 cells (compare lanes 1 and 2 in Figure 6), when applied to extracts of A9 cells which express the mutant hu-p55-TNF-Rs, they did precipitate some of the murine receptors along with the human receptors. The latter were easily distinguishable by their full length (lanes 3–6 in Figure 6B, particularly evident in lane 6). Longer exposure of the autoradiograms of the immunoprecipitated proteins analysed by SDS–PAGE also

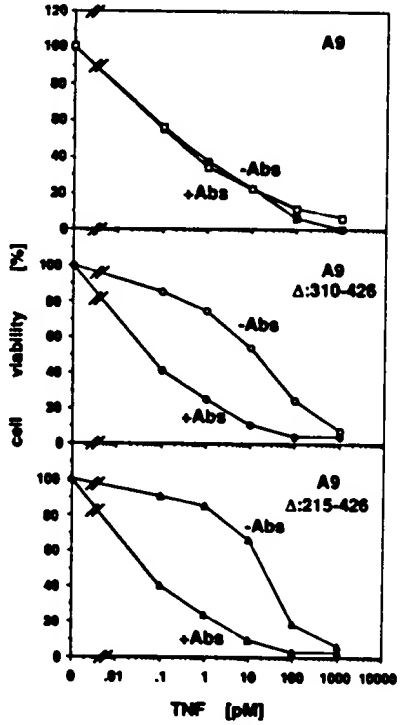

Fig. 5. Treating A9 cells expressing mutant hu-p55-TNF-Rs with antibodies to the hu-p55-TNF-R restores sensitivity to the cytocidal effect of TNF. The cytocidal effect of TNF, applied together with CHI (50 µg/ml) to A9 cells (top panel), to A9 cells expressing the Δ:310-426 mutant (middle panel) and to A9 cells expressing the Δ:215-426 mutant (bottom panel), was examined in the presence (solid symbols) and absence (empty symbols) of antibodies to the hu-p55-TNF-R (monoclonal antibodies 18 and 20, 10 µg/ml of each). Similar sensitization by the antibodies to the cytocidal effect of TNF was observed in A9 cells expressing the Δ:244-426 mutant.

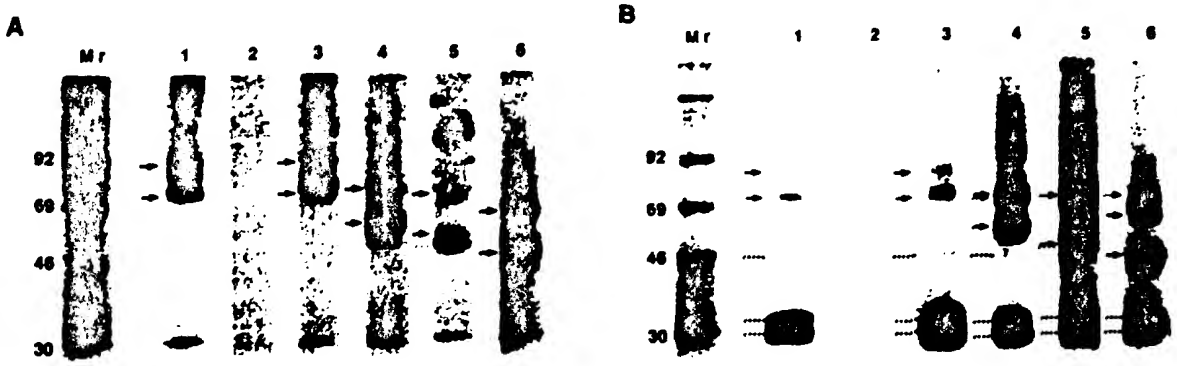

Fig. 6. SDS–PAGE analysis of the full-length and truncated forms of the hu-p55-TNF-Rs, tagged by cross-linking to [^{125}I]TNF. The receptors were immunoprecipitated from cell extracts with (A) or without (B) prior acidification. (1) HeLa cells. (2) A9 cells. (3) A9 cells expressing the wild type hu-p55-TNF-R. (4) A9 cells expressing the Δ:310-426 mutant. (5) A9 cells expressing the Δ:244-426 mutant. (6) A9 cells expressing the Δ:215-426 mutant. (Mr) molecular weight markers (Amersham). Bands whose sizes correspond to the expressed human receptors, tagged by cross-linking to either one or two labelled TNF molecules, are denoted with solid arrows (sizes of 72 and 89 kDa for the full-length receptor, 59 and 76 kDa for the Δ:310-426 mutant, 51 and 68 kDa for the Δ:244-426 mutant and 48 and 65 kDa for the Δ:215-426 mutant). The labelled bands whose size correspond to the full-length murine receptors, cross-linked to TNF (72 kDa); are denoted by empty arrows and the bands which correspond to the TNF monomer, and to the cross-linked dimers and trimers of TNF (17, 34 and 51 kDa) by stippled lines.

revealed slowly migrating labelled bands which could be suspected, by their size, to consist of hetrotrimers composed of the murine receptor, the human receptor and TNF, cross-linked to each other (upper parts of the gels in Figure 6). These observations suggest that the TNF receptors exist in aggregates containing receptors of both human and murine origin. In accord with this, lowering the pH of the cell extracts prior to immunoprecipitation, to dissociate non-covalently linked receptors, resulted in decreased precipitation of the murine, but not of the human receptors (compare panels A and B in Figure 6). Conceivably, the aggregation occurs in response to the TNF, which was applied to the cells for tagging the receptors.

Shedding of the full-length and truncated forms of the human p55 TNF receptor by the rodent cells

The formation of a soluble form of the hu-p55-TNF-R in A9 cells expressing the full-length human receptor was examined using an immunoassay. Slow, spontaneous shedding of the hu-p55-TNF-R was observed which was not increased by application of TNF, but was markedly enhanced when the cells were treated with the tumour promoting phorbol diester 4β-phorbol-12-myristate-13-acetate (PMA) (Figure 7, upper panel). PMA was reported to down-regulate the expression of the cell surface TNF receptors (Holtmann and Wallach, 1987; Unglaub *et al.*, 1987). The kinetics of decrease in cell surface TNF receptors in cells treated with PMA correlated with the formation of the soluble form of these receptors (compare upper and lower panels in Figure 7). Both showed significant changes within a few minutes of application of PMA, indicating that the effect of PMA on formation of the soluble receptors does not reflect an enhancement of synthesis of the TNF receptors, but stimulation of their proteolytic cleavage. Indeed, the effect of PMA could not be blocked by inhibiting protein synthesis in the A9 cells using cycloheximide (not shown).

HeLa cells exhibit a similar spontaneous shedding of the p55-TNF-R which is also enhanced by PMA and not affected by TNF (not shown).

Study of the formation of the soluble form of the hu-p55-TNF-R in A9 cells expressing the truncated receptors

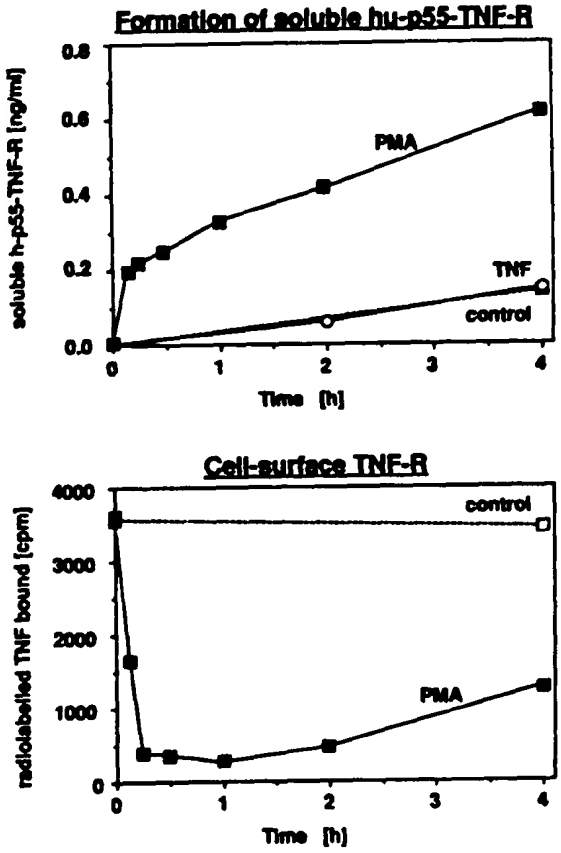

Fig. 7. Kinetics of the release of the soluble form of the hu-p55-TNF-R (upper panel) and of the decrease in expression of cell surface TNF-Rs (lower panel) in A9 cells expressing the wild-type human receptor, without addition (□), or after treatment with PMA (32 nM, ■) or TNF (1 nM, ○). After incubation for the indicated time periods, the concentration of the soluble form of the hu-p55-TNF-R in the culture media and specific binding of ^{125}I-labelled TNF to the cells were quantified as described in Materials and methods. TNF, at a concentration of 1 nM, did not interfere with the immunoassay of the soluble TNF-R.

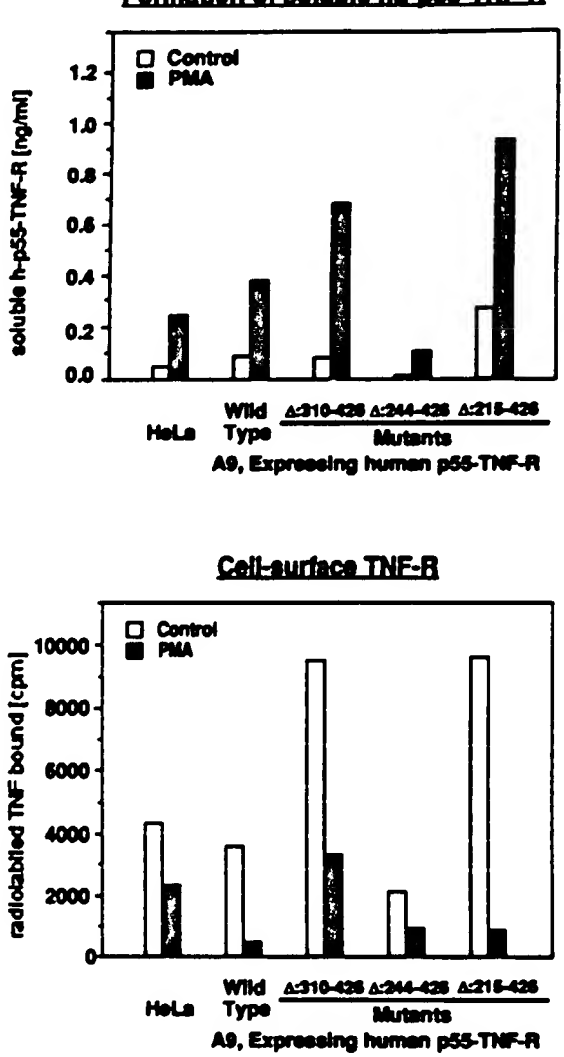

Fig. 8. Receptor shedding (upper panel) and TNF binding (lower panel) in HeLa cells and in A9 cells expressing the wild-type or cytoplasmic deletion mutant hu-p55-TNF-Rs. The amounts of the soluble form of the hu-p55-TNF-R in the culture media and the specific binding of ^{125}I-labelled TNF to the cells were quantified as described in Materials and methods after 2 h incubation of the cells at 37°C in the presence or absence of PMA (32 nM).

revealed, in all, spontaneous shedding of the receptor and enhancement of the shedding with PMA, associated with a decrease in the amounts of the cell surface receptors (Figure 8). Neither the spontaneous shedding of the receptor, nor the extent of its enhancement by PMA, seemed to be affected by the extent of cytoplasmic truncation of the receptor; values were roughly proportional to the amounts of human receptor expressed by the particular clone examined (compare Figure 8 upper and lower panels).

Discussion

Expression of the human p55 TNF receptor (hu-p55-TNF-R) in rodent cells provides a convenient system for exploring its mechanisms of action. As recently disclosed by the cloning of the cDNA for the hu-p55-TNF-R (Loetscher *et al.*, 1990; Nophar *et al.*, 1990; Schall *et al.*, 1990) and of the cDNAs for the rat and mouse p55-TNF-Rs (Himmler *et al.*, 1990; Barrett *et al.*, 1991; Lewis *et al.*, 1991), there is considerable conservation of structure between the human and rodent receptors. Consistent with that, there is no species specificity barrier for at least part of the receptor functions. The mouse receptor binds human TNF with an affinity similar to that of the human receptor (Lewis *et al.*, 1991) and, as demonstrated in the present study, the human receptor can signal a cytocidal effect within rodent cells and is subject, just as in human cells, to both spontaneous and inducible cleavage. The human and rodent receptors are, however, immunologically distinct. The antigenic differences between the receptors on the one hand, and the lack of species specificity in their function on the other, permitted us to examine the function of mutant human receptors by expressing them in rodent cells and to differentiate between their function and that of the endogenous receptors, using mimetic antibodies specific to the human receptors.

In the present study, by expressing mutants of the hu-p55-TNF-R in rodent cells, we studied the effect of cytoplasmic truncation on two different activities of this receptor: signalling for the cytocidal effect of TNF and cleavage of the receptors which results in formation of their soluble forms. Our findings, which are summed up in Figure 3, relate to two central questions regarding the function of the TNF receptors. Firstly, how do the TNF receptors signal intracellularly to induce the effects of TNF? Secondly, what is the relationship between the formation of the soluble forms of the TNF receptors and other activities of these receptors—more specifically, is the generation of the soluble receptors a 'side product' of the signalling for the effects of TNF or is it a product of mechanisms which are specific for the production of these soluble forms?

C-terminal truncation of the intracellular domain of the hu-p55-TNF-R by 53% abolished its signalling activity. Yet even further truncation, by as much as 96% of this domain, did not prevent the shedding of the receptor in response to PMA. The effects of cytoplasmic deletions on the signalling activity of the receptors indicate that the signalling activity resides in the cytoplasmic domain of the receptor, thus distinguishing the p55 TNF-R from receptors like that of IL6, which transduce its signals to the cell interior indirectly—not by its own intracellular domain, but by the intracellular domain of an effector molecule associated with this receptor (Taga *et al.*, 1989).

What signals the TNF-Rs generate are not known. However, characterization of the way TNF-like effects are initiated by antibodies against the p55 TNF-R (Espevik *et al.*, 1990; Engelmann *et al.*, 1990a) indicate that the mere clustering of molecules of this receptor suffices for the triggering of its signalling activity (Engelmann *et al.*, 1990a). Furthermore, two observations in the present study are consistent with the notion that clustering of the p55 TNF-R is not only a sufficient, but a necessary condition for the triggering of its activities, and occurs as part of the process of its stimulation by its natural ligand, TNF. Analysis of the molecular size of the transfected receptors, after tagging with radiolabelled TNF (Figure 6), revealed that these receptors are associated with the murine receptors. Conceivably, although confirmation is needed, this association is a consequence of TNF binding. A functional role for

this aggregation in cell response t TNF is indicated by the marked inhibitory effect of the truncated forms of the hu p55-TNF-R on the response of rodent cells to the cytocidal effect of TNF (Figure 4, right panel). This inhibition suggests some kind f intermolecular interactions between the recept rs. Its most likely interpretation is the occurrence of 'futile' clustering: association of the endogenous, full-length receptors with the truncated human receptors, which greatly exceed them in number, resulting in formation of non-functional complexes. Indeed, applying antibodies against the hu-p55-TNF-R on cells that express the truncated receptors, thus blocking the interaction of these receptors with TNF, resulted in recovery of the sensitivity f these cells to the TNF effect (Figure 5). In receptors with tyrosine kinase activity, triggering of signalling upon receptor aggregation could be ascribed to intermolecular cross-phosphorylation of the receptor molecules. The signalling activity itself was shown to reflect phosphorylation of some other cellular substrates by the receptors (reviewed in Ullrich and Schlessinger, 1990). The p55 TNF-R appears to be devoid of protein kinase activity (Loetscher *et al.*, 1990; Nophar *et al.*, 1990; Schall *et al.*, 1990). Its amino acid sequence provides no indication of any other way by which it can signal. The loss of its ability to initiate the cytocidal effect upon deletion of the 125 C-terminal amino acids suggests that the structural element(s) which signal for the cytocidal effect reside, at least partly, within this C-terminal region. Study of receptor mutants with more restricted deletions of sequences should facilitate identification of these molecular structures and clarify whether these same structures or others are involved in the induction of other, non-cytocidal effects of TNF.

The occurrence of inducible shedding in receptor molecules which are deficient in signalling activity, indicates that the shedding and signalling mechanisms are distinct. The fact that TNF, which triggers the signalling, has no effect on the shedding, further supports this notion. These observations suggest that the process of cleavage of the TNF receptors which results in formation of their soluble forms, is not a mere 'side product' of the signalling for the TNF effects. Rather, it appears to occur by mechanism(s) independent of other functions of the receptors, directed specifically to inhibit TNF action both by decreasing the amounts of the cell surface receptors and by creating soluble forms, which are inhibitory to TNF function.

The finding that truncation of the intracellular domain has no effect on the shedding of the p55 TNF-R, nor on its enhancement by PMA, is consistent with a prior study on the mechanism of inducible shedding of the receptor for CSF-1 (Downing *et al.*, 1989). Evidence was presented in that paper that PMA enhances shedding of the CSF-1 receptor, through activation of protein kinase C, not by phosphorylation of the receptor itself but by activation of the protease(s) which cleave it. This seems to be the case also for the effect of PMA on the shedding of the p55 TNF-R, shown in the present study to be maintained even after removal of almost all potential phosphorylation sites in the intracellular domain. A clue as to the identity of the protease which cleaves the p55 TNF-R and to the modes of its regulation can be gained by defining the exact site of receptor cleavage. This information may be reached by analysis of additional receptor mutants, looking specifically for mutants which will fail to exhibit induced shedding.

Materials and methods

Construction of mutant p55 TNF receptors

The cDNA of the human p55 TNF-R (Nophar *et al.*, 1990) was digested with *Ban*II and *Nhe*I, resulting in removal of large parts of the 5' and 3' non-coding regions. Mutants Δ:310-426 and Δ:215-426 (Figure 3) were generated by oligonucleotide directed mutation of this shortened form of the cDNA, using the 'Altered Sites' mutagenesis kit of Promega. Stop codons were introduced after Leu309 [mutant Δ:310-426; amino acids in the receptor are numbered according to Loetscher *et al.* (1990)] using the oligonucleotide 5'-CCCCAACCCCCTCTAGAAGTGGGAGG-3', and after Leu214 (mutant Δ:215-426), using the oligonucleotide 5'-AGTCCAAGCTCTAGACCA-TTGTTTGTGG-3'. The wild-type and mutated cDNAs were introduced into the eukaryotic expression vector pMPSVEH (Artelt *et al.*, 1988, kindly provided by Dr H.Hauser, GBF, Braunschweig, FRG), which contains the myeloproliferative sarcoma virus promoter, an SV40 intron and the SV40 polyadenylation signal. For the generation of the Δ:244-426 mutant, the expression vector containing the wild-type cDNA was digested with *Hin*dIII. The resulting 3.9 kb fragment was isolated and then, after filling in of the protruding ends religated, thus replacing amino acid 244 by a stop codon.

Expression of the wild-type and mutant receptors in cultured cells

Cells of the murine A9, L929, NIH 3T3 and the hamster BHK lines were cultured with Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (growth medium). The cells were transfected with the expression constructs encoding the wild-type and mutant receptors, together with the neomycin resistance conferring plasmid pSV2neo, using the calcium phosphate precipitation method (Graham and van der Eb, 1973). After 10–14 days selection in growth medium containing 500 μg/ml G418 (Sigma), resistant colonies were isolated and examined for expression of the human p55 TNF-R (hu-p55-TNF-R) by measuring TNF binding to the cells.

Quantification of the cytocidal effect of TNF and of antibodies to the hu-p55-TNF-R

Cells were seeded into 96-well plates, 24 h before the assay, at a density of 30 000 cells per well. The growth medium was then exchanged with 100 μl growth medium containing cycloheximide (CHI, 25 μg/ml for the HeLa cells and 50 μg/ml for all other cell types and TNF or antibodies against the hu-p55-TNF-R). After further 11 h incubation at 37°C, viability of the cells was assessed in a neutral red uptake assay as described before (Wallach, 1984).

Determination of TNF binding to cells and of TNF internalization and degradation

Recombinant human TNF-α (TNF, 6×10^7 U/mg of protein), produced by Genentech Co., San Francisco, CA, was kindly provided by Dr G.Adolf, of the Boehringer Institute, Vienna, Austria. The TNF, was radiolabelled with chloramine T to a specific radioactivity of 2000 μCi/mmol (Israel *et al.*, 1986). To determine the binding and internalization of TNF cells were seeded into 15 mm tissue culture plates at a density of 2.5×10^5 cells/plate. After 24 h incubation at 37°C, the plates were transferred to ice, the growth medium was removed and radiolabelled TNF was applied at the concentration of 0.1 nM, either alone or with a 1000-fold excess of unlabelled TNF, in 200 μl PBS (0.154 M sodium chloride plus 10 mM sodium phosphate, pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA and 0.02% NaN_3 (binding buffer). After 3 h of incubation on ice, the cells were rinsed and then detached by incubation in PBS containing 5 mM EDTA. Cell-bound radioactivity was determined in a γ-counter. The data were analysed using the LIGAND program (Munson and Rodbard, 1980). Alternatively, after the binding of radiolabelled TNF the cells were incubated in growth medium at 37°C to follow the uptake and degradation of the cell-bound TNF. At various times, plates were transferred to an ice bath and the cell-bound TNF was dissociated by low pH treatment (incubated for 5 min with ice-cold 50 mM glycine HCl buffer, pH 3.0, followed by rinsing three times with binding buffer). The amount of internalized TNF was determined by detaching the cells and counting them in a γ-counter as described above. The extent of degradation of TNF was assessed by measuring the amount of trichloroacetic acid-soluble radioactivity in the cell growth medium.

Chemical cross-linking of radiolabelled TNF to the receptors

Cells were detached by incubation in PBS containing 5 mM EDTA, rinsed with PBS containing 0.5% BSA and 0.02% NaN_3 (binding medium), and suspended in aliquots of 5×10^7 cells in 1 ml binding medium containing 0.2 nM radiolabelled TNF. After incubation with occasional shaking for 4 h on ice, the cells were washed once with PBS and incubated for 20 min

in the same buffer containing 1 mM bis(sulphosuccinimidyl)suberate (Pierce). Cross-linking was stopped by adding Tris–HCl and glycine–HCl, pH 7.4 (both to a final concentration of 100 mM) followed, after 10 min incubation on ice, by two washings with PBS. The cells were then extracted for 1 h at 4°C, using 600 μl of a lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% deionized Triton X-100, 1 μg/ml leupeptin (Sigma) and 1 mM-phenylmethylsulphonyl fluoride (Sigma). After centrifugation for 30 min at 10 000 *g*, the cell extracts were divided into two equal portions. One was acidified by adding 90 μl 1 M glycine–HCl buffer, pH 2.5, and, after incubation for 1 h on ice, neutralized with 30 μl 1 M NaOH. Monoclonal antibodies against the hu-p55-TNF-R (numbers 18 and 20, Engelmann *et al.*, 1990a, 12 μg of each) were added to both portions of the extracts. After further incubation for 12 h at 4°C, 20 μl protein A–Sepharose beads (Pharmacia), equilibrated with PBS, were added. Following incubation for 60 min at 4°C, they were washed three times with the lysis buffer containing 2 M KCl, and twice with PBS. The beads were resuspended in 15 μl sample buffer containing 4% (w/v) SDS and 6% (v/v) β-mercaptoethanol and boiled for 3 min. The supernatant was analysed by SDS–PAGE (10% polyacrylamide) followed by autoradiography.

Measurement of the shedding of the soluble form of the hu-p55-TNF-R

Cells were seeded 24 h before the assay into 15 mm tissue culture plates at a density of 2.5×10^5 cells/plate. At time zero, the medium was replaced with 300 μl growth medium containing the indicated additives (PMA, PMA plus CHI or TNF). After further incubation for various time periods, samples of the medium were collected and centrifuged at 3000 *g* for 5 min to sediment the cells and cell debris. Content of the soluble form of the hu-p55-TNF-R in the supernatants was determined by a two site capture ELISA, using a mouse monoclonal antibody and rabbit antiserum against this protein as described elsewhere (Aderka *et al.*, 1991).

In all experiments, the data presented are mean values of duplicate determinations. Variation between duplicate samples was in all cases < 10% of the mean.

Acknowledgements

We are grateful to Dr Giampiero De Luca and Dr Umberto Canosi and his group, of the Istituto di Ricerca Cesare Serono, Ardea, Italy, for their hospitality and generous help which allowed the continuity of this study at the time of the Gulf war. Our thanks are also due to Messrs Adela Diberman and Ayala King for expert technical assistance, to Dr Hansjörg Hauser for useful advice and for providing the pMPSVEH vector and to Drs Helmut Holtmann and Dan Aderka and Ms Ayala King for helpful comments on the manuscript. This work was supported by a grant from Inter-Lab Ltd, Ness-Ziona, Israel, as well by a grant from the National Council for Research and Development, Israel and the German Cancer Research Centre. C.B. and O.K. are recipients of fellowships of the Minerva foundation (Germany). Portions of the work, carried out by C.B. during his stay at the Weizmann Institute, will be submitted as part of his thesis for the requirements of a Ph.D. degree from the School of Medicine, Hannover, Germany.

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Received on October 11, 1991; revised on November 28, 1991

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Journal code: B53. ISSN: 1078-7852.

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KEYWORDS:
cytokines
macrophages
membrane
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signaling

ABBREVIATIONS:

BSA	bovine serum albumin
FCS	fetal calf serum
LPS	lipopolysaccharide endotoxin
MM6	the Mono Mac 6 cells
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl-fluoride
SDS	sodium dodecyl sulfate
TNF	tumor necrosis factor

Endorsed by Bruce Beutler and Daniela Männel.

Accepted May 30, 1995.

Éva Pócsik was on a three-month fellowship of a Yamagiwa-Yoshida Memorial International Cancer Study Grant provided by the National Union Against Cancer.

Address correspondence to Dr. David Wallach, Department of Membrane Research and Biophysics, Weizmann Institute of Science, Rehovot 76100, Israel.

RESEARCH ARTICLE

Phosphorylation of the 26 kDa TNF Precursor in Monocytic Cells and in Transfected HeLa Cells

Éva Pócsik, Ernô Duda, and David Wallach

Laboratory of Cellular Immunology, National Institute of Haematology, Blood Transfusion and Immunology, Budapest (E.P.), Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged (E.D.), Hungary; Department of Membrane Research and Biophysics, Weizmann Institute of Science, Rehovot, Israel (D.W.)

Tumor necrosis factor (TNF) functions both as a soluble molecule and as a cell surface 26 kDa transmembrane protein, from which the soluble form is proteolytically derived. The 26 kDa TNF molecules isolated from ^{32}P labeled HeLa cells that had been transfected with the cDNA of a partially cleavable TNF mutant were found labeled. Phosphorylated 26 kDa TNF molecules could also be isolated from human LPS stimulated monocytic Mono Mac 6. Phosphoaminoacid analysis revealed that the labeled phosphate is bound to serine residues. No label was found incorporated in soluble 17 kDa TNF, indicating that the phosphorylated residue(s) of membrane-associated TNF occur in the cytoplasmic portion of the molecule. Phosphorylation of the intracellular domain of the 26 kDa TNF molecules may play a role in the regulation of expression or proteolytic processing of TNF, modulate TNF bioactivity, or take part in intracellular signaling by cell-surface TNF. © *1995 Wiley-Liss, Inc.*

INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic cytokine that plays a central role in the induction of inflammation. Its wide range of effects include cytotoxicity, stimulation of cell growth, and induction of changes in cell differentiation patterns and various immune activities [1,2]. It is primarily produced in mononuclear phagocytes following their stimulation with bacterial components, such as lipopolysaccharide (LPS), or viruses, or multicellular parasites. TNF molecules are initially produced in the form of 26 kDa β-transmembrane proteins with a signal

peptide of 76 amino acid residues [2]. These transmembrane molecules may remain on the surface of the cells that produce them or are proteolytically processed, yielding soluble 17 kDa TNF molecules [3–5].

Both the cell surface and soluble forms of TNF can trigger effects characteristic of this cytokine in target cells by binding to the same two species of TNF receptors [3,4,6–11]. However, there are some differences in their mode of action resulting from the differences in their structure and physical state. The soluble form of TNF acts at a multiplicity of sites, adjacent to its formation site as well as distant from it, as is the case with other endocrine regulators, while the function of cell-bound TNF is limited to the vicinity of the TNF producing cell. In addition, the mechanism of action of cell surface TNF differs from that of the soluble form in terms of the extent of influence of the individual TNF-producing cell on the nature of the effects of the cytokine. Unlike soluble TNF and other endocrine mediators, whose mode of action is largely independent of their way of formation, cell-bound TNF molecules act in a way which dictates a direct link between TNF production and function. The location of the effector cell, the effectivity of TNF production, and, perhaps, also the way in which the cell presents TNF on its surface, determine the identity of the target cell and its mode of response. There also seem to be some differences in the nature of the effects induced by the two molecular forms of TNF [7,12], suggesting that they can trigger different signaling activities.

In view of the distinctive features of the mechanism of action of cell-bound TNF, some types of control mechanisms specifically regulating the action of these molecules are likely to exist. The intracellular domain of the membrane-associated TNF molecules is likely to serve such a role since it is accessible to modulation by intracellular mechanisms. Although the intracellular domain of the TNF molecule has no direct involvement in TNF receptor binding its sequence is highly conserved among different animal species, suggesting that it has an important function [reviewed in 13,14]. One possible way by which signals within the TNF producing cells can affect the function of cell surface TNF is described in the present study. Evidence is presented that the intracellular region of transmembrane TNF is subject to phosphorylation by protein kinase(s).

MATERIALS AND METHODS

Reagents

Cell culture media and supplements were purchased from GIBCO (Grand Island, NY); bovine insulin, lipopolysaccharide (LPS) (from *Salmonella* Minnesota), phenylmethylsulfonylfluoride (PMSF), leupeptin, and diaminobenzidine-tetrahydrochloride from Sigma Chemical Co. (St. Louis, MO); protein G-Sepharose (fast flow) from Pharmacia Fine Chemicals (Piscataway, NJ); [^{35}S] met and carrier-free [^{32}P] orthophosphoric acid, and the Amplify intensifying reagent were purchased from Amersham Corp. (Arlington Heights, IL). The nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA). A mouse monoclonal antibody specific to human TNF (TNF-1) and polyclonal sheep and rabbit anti-human TNF sera were developed in our laboratories. Human IgG, FITC-labeled goat anti-mouse IgG F(ab)'2, nonimmune sheep serum, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Bio-Makor (Rehovot, Israel).

Cell Culture

Human acute monocytic leukemia Mono Mac 6 (MM6) cells [15] were obtained from the German Collection of Microorganisms and Cell Cultures. They were grown at a cell density range of $0.3-1 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM Na-pyruvate, 1% nonessential amino acids, 9 μg/ml bovine insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Epithelioid cervical carcinoma HeLa cells [16] were obtained from the American Type Culture Collection (Rockville, MD). HeLa-M9 cells are a clone of HeLa cells which constitutively express, under control of the SV40 promoter, a TNF mutant cDNA in which the arginine at position +2 and the serine at position of +3 are substituted with threonines (the pstA11 construct). These mutations cause an about tenfold reduction in the cleavage rate of 26 kDa TNF (A. Mai and E. Duda, unpublished study). The HeLa and HeLa-M9 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin.

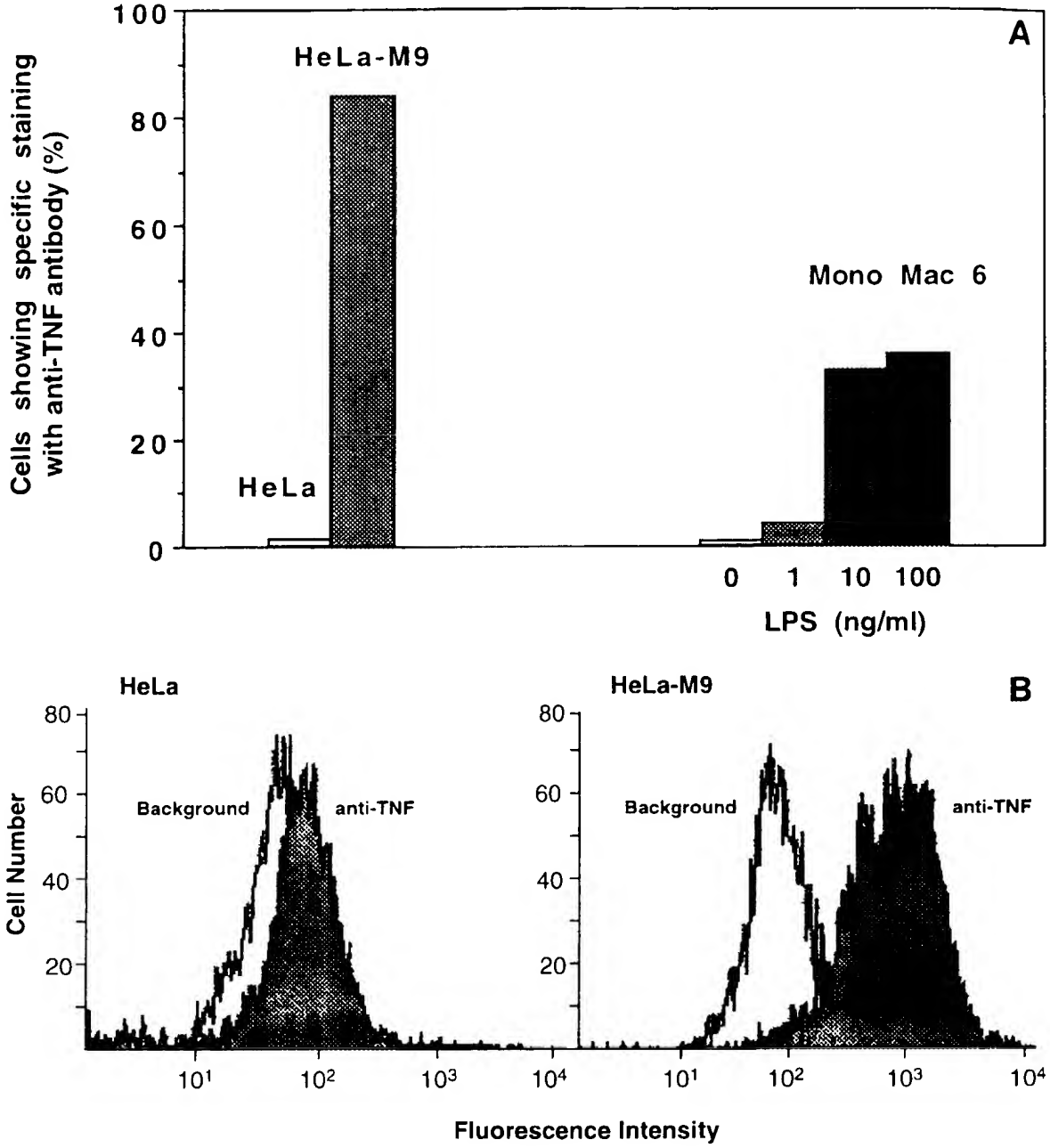

FIGURE 1

Cell surface TNF in HeLa-M9 cells and in LPS-treated MM6 cells. A: Flow cytometric analysis data of cell surface TNF expression in HeLa cells, HeLa-M9 cells, and MM6 cells treated for 2hr with LPS at the indicated concentrations. B: FACS profiles of HeLa and HeLa-M9 cells stained with anti-TNF antibody. Background denotes cells stained in the absence of anti-TNF antibody.

Indirect Immunofluorescence

Indirect immunofluorescence analysis was performed as described elsewhere [17]. Briefly, samples of 5×10^5 cells were incubated for 30 min at 4°C in the presence of 10 μg/ml mouse monoclonal antibody against human TNF (TNF-1) in phosphate buffered saline (PBS), containing 2 mg/ml BSA, 2 mg/ml human IgG, and 0.1% sodium azide, and then with FITC-conjugated goat anti-mouse IgG F(ab)'2, followed by fixation with 1% formaldehyde. Samples of 5,000 cells were analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Metabolic Labeling

Labeling of cells with [^{35}S] met or [^{32}P] orthophosphate was performed by incubation in met free or phosphate free medium, supplemented with 5 or 10% FCS that had been dialyzed against either PBS or 0.9% NaCl, respectively. Unless otherwise indicated, [^{35}S] met and [^{32}P] orthophosphate were added to the cells for 2.5 hr, at concentrations of 100 μCi/ml and 50 μCi/ml, respectively. Labeling with [^{35}S] met was performed after a 15 min preincubation in met free medium. In the experiments with LPS-stimulated MM6 cells, treatment with LPS was performed simultaneously with the metabolic labeling.

Immunoprecipitation and Gel Electrophoresis

Immunoprecipitation was performed using sheep anti-TNF antiserum or, as a control, nonimmune sheep serum, at a dilution of 1:200. To specifically immunoprecipitate cell surface TNF, the antisera, diluted in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% sodium azide, were added to the cells prior to their lysis. The cells were incubated for 30 min with the antisera and then rinsed with ice-cold PBS. To also immunoprecipitate intracellular TNF molecules, the antisera were directly added to the cell lysate, for a period of 2 hr. Cell lysis was performed by incubating the cells for 30 min at a cell concentration of 1×10^7 cells/ml in a lysis buffer comprised of 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.02% sodium azide, 0.1 mM PMSF, and 2 μg/ml leupeptin, followed by centrifugation at 12,000*g* for 15 min to sediment insoluble material. In the [^{32}P] labeling experiments, the lysis buffer was supplemented with 100 μM Na-orthovanadate, 1 mM EGTA, and 50 mM NaF. Precipitation of the antibodies was done using Protein G-Sepharose beads. All immunoprecipitation steps were performed at 4°C. The immunoprecipitated proteins were analyzed by SDS-PAGE under reducing conditions (12% acrylamide). Gels used for the analysis of [^{35}S] labeled proteins were treated with the Amplify intensifying reagent.

Western Analysis

Following SDS-PAGE analysis, proteins were Western-blotted to nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany). The blots were probed either with rabbit anti-TNF antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG and developed with diaminobenzidine-tetrahydrochloride, or with [^{125}I] rabbit anti-TNF antibody labeled with the Iodogen reagent [18] using 1×10^7 CPM/blot.

Phosphoamino Acid Analysis

To identify the phosphorylated amino acid residue(s) in TNF, [^{32}P] labeled TNF was isolated from extracts of HeLa-M9 cells that had been labeled by incubation for 5 hr in growth medium containing 500 μCi [^{32}P] orthophosphate/ml. The labeled amino acids in the protein were identified as described [19]. Briefly, following immunoprecipitation and SDS-PAGE analysis, the protein was blotted onto Immobilon PVDF membrane (Millipore, Bedford, MA). The 26 kDa TNF band, identified by autoradiography, was excised from the membrane and hydrolyzed in 6 N HCl for 1 hr at 110°C. The resulting hydrolysate, to which 0.3 μg of each nonlabeled phosphoamino acid marker was added, was fractionated by high voltage two-dimensional thin layer chromatography. The position of the labeled residues, detected by 4 day exposure for autoradiography, was compared with those of the nonlabeled residues, as determined by ninhydrin staining.

RESULTS

Two cellular systems were employed in this study for characterizing the 26 kDa TNF precursor: i) HeLa cells that constitutively express transfected cDNA for mutated TNF, exhibiting reduced processing rates; and ii) cells of the human monocytic leukemia line Mono Mac 6 (MM6), which produce the TNF precursor upon LPS stimulation [20]. As determined by fluorescence-activated cell sorting (FACS) analysis using monoclonal anti-TNF antibody, both the TNF-transfected HeLa cells (HeLa-M9 cells) and the MM6 cells express TNF on their surface (Fig. 1). In the MM6 cells, treatment with LPS resulted in enhanced cell surface TNF expression, showing maximal effect at 10–100 ng of LPS per ml. The signal observed in FACS analysis was not affected by treating the cells with high salt concentration following fixation, indicating that the TNF molecules are integral to the cell membrane and not soluble molecules adsorbed to the cells (data not shown).

Immunoprecipitation studies revealed that

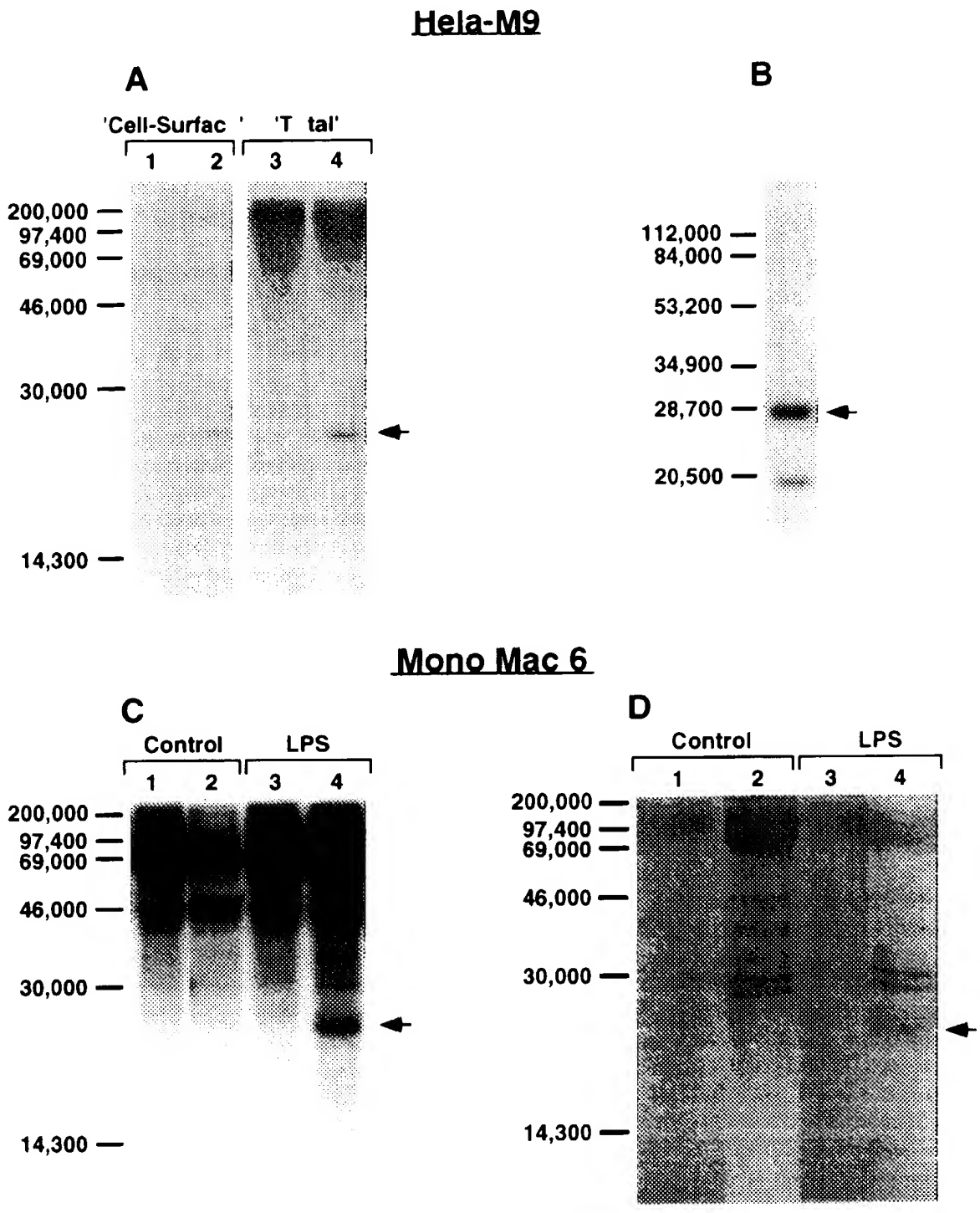

FIGURE 2

SDS-PAGE and Western blotting analysis of TNF expressed in HeLa-M9 and LPS-treated MM6 cells. TNF expression in HeLa-M9 cells (A,B) and MM6 cells (C,D). A: Proteins were immunoprecipitated with anti-TNF antibody (lanes 2 and 4) or with control serum (lanes 1 and 3) from lysates of HeLa-M9 cells that had been metabolically labeled with [^{35}S] met. Immunoprecipitation was performed by applying the antibodies either before cell lysis, followed by removal of nonbound antibodies, to specifically detect the cell surface TNF (lanes 1, 2: 'cell surface'); or after cell lysis, to also detect intracellular TNF molecules (lanes 3, 4: 'total'). B: Western blotting analysis of proteins in the lysate of HeLa-M9 cells that react with anti-TNF antibody. C: The proteins immunoprecipitated with anti-TNF antibody (lanes 2 and 4) or with control serum (lanes 1 and 3) from lysates of MM6 cells metabolically labeled with [^{35}S] met and treated (lanes

the cell-surface protein recognized by anti-TNF antibodies is the 26 kDa TNF precursor. Two methods of immunoprecipitation were employed: i) anti-TNF antibodies were incubated with TNF-producing cells prior to cell lysis, thus allowing the antibodies to interact only with cell-surface TNF molecules; or ii) anti-TNF antibodies were added to the cells following lysis, permitting them to also interact with intracellular TNF molecules. Using both immunoprecipitation methods, we observed specific recognition of the 26 kDa protein in [^{35}S] met labeled HeLa-M9 cells. Much greater amounts of the protein were immunoprecipitated if antibodies were added after cell lysis than before lysis, suggesting that most of the 26 kDa TNF molecules occur within the HeLa-M9 cells (compare lanes 3, 4 to 1, 2 in Fig. 2A). Western blot analysis revealed that, in addition to the 26 kDa TNF molecules, lysates of HeLa-M9 cells contain some 17 kDa TNF molecules (Fig. 2B). These molecules could not be detected by labeling with [^{35}S] met since the 17 kDa TNF does not contain methionine. We also observed 26 kDa TNF in lysates of LPS-stimulated MM6 cells (Fig. 2C, D), although in much lower amounts. TNF molecules could be detected when the antibodies were added to the MM6 cells after lysis but not before lysis (data not shown). TNF was not detectable in nonstimulated MM6 cells (lanes 1 and 3 in Fig. 2C, D), or in HeLa cells which had not been transfected with TNF cDNA (not shown).

In both the HeLa-M9 cells and LPS activated MM6 cells, growth in the presence of [^{32}P] resulted in incorporation of label in the 26 kDa TNF precursor molecules (Fig. 3). As in the [^{35}S] met labeling experiments, the amount of cell surface [^{32}P] radiolabeled TNF in the MM6 cells was too low to be detected, though we did find radiolabeled TNF in the whole cell lysate. Yet, we could isolate [^{32}P] labeled TNF molecules in the HeLa-M9 cells using both ways of immunoprecipitation (Fig. 3A, lanes 2 and 4), indicating that the cell surface TNF molecules are phosphorylated. No label could be discerned in the 17 kDa form of TNF (compare Fig. 3A, lane 4 to Fig. 2B).

Phosphoamino acid analysis showed that the label in the 26 kDa TNF molecules expressed in HeLa-M9 cells is bound to serine residues (Fig. 4).

DISCUSSION

This study employed cellular systems that provide effective expression of the membrane bound form of TNF, to allow study of the molecular properties of this protein which is normally present in very low amounts. The MM6 monocytic leukemia cells were chosen since, in contrast to some other cultured cells of monocytic origin, LPS-stimulated TNF production in them is not accompanied by induced TNF shedding. Thus, the transmembrane form of TNF is effectively accumulated in these cells [20]. Indeed, 26 kDa TNF molecules were easily detected in lysates of LPS-treated MM6 cells. However, the amounts of cell surface TNF molecules in these cells were too low to allow their detection by metabolic labeling (although they could be detected by FACS analysis). We therefore decided to use an artificial experimental system where TNF was expressed in HeLa cells under the control of a strong promoter. To further enhance the expression of the precursor TNF molecules, we used a mutant TNF molecule that cannot be processed effectively. The change introduced by the mutation (substitution of the arginine and serine at positions +2 and +3 with threonines) was milder than applied in a previous study (deletion of amino acids 1–12 in TNF [4]), to minimize distortion of normal TNF function. This change does not fully prevent the proteolytic cleavage of TNF, but does result in the accumulation of 26 kDa TNF molecules, both intracellularly and on the cell surface.

We found that the 26 kDa TNF molecules are phosphorylated. The high amounts of TNF in the transfected HeLa cells permitted further studies in which we learnt that i) both the cell surface and intracellular 26 kDa TNF molecules are phosphorylated, ii) the phosphorylated residues in TNF are serines, and iii) the soluble 17 kDa TNF molecules

FIGURE 2 (Continued)

3 and 4) or not treated (lanes 1 and 2) with LPS (100 ng/ml for 2 h). D: Western blotting analysis of the binding of anti-TNF (lanes 2, 4) or a control (lanes 1, 3) antibody, to the proteins in lysates of MM6 cells that had been treated (lanes 3, 4) or not treated (lanes 1, 2) with LPS as above. Development of the Western blots was performed using radiolabeled anti-TNF antibody in B, and enzymatically in D, as described in Materials and Methods. The protein samples applied for analysis were from the following number of cells: in A, lanes 1 and 2—1.8×10^6, lanes 3 and 4—0.6×10^6; in B, 1.8×10^6; in C, 2×10^6 and in D, 1×10^6.

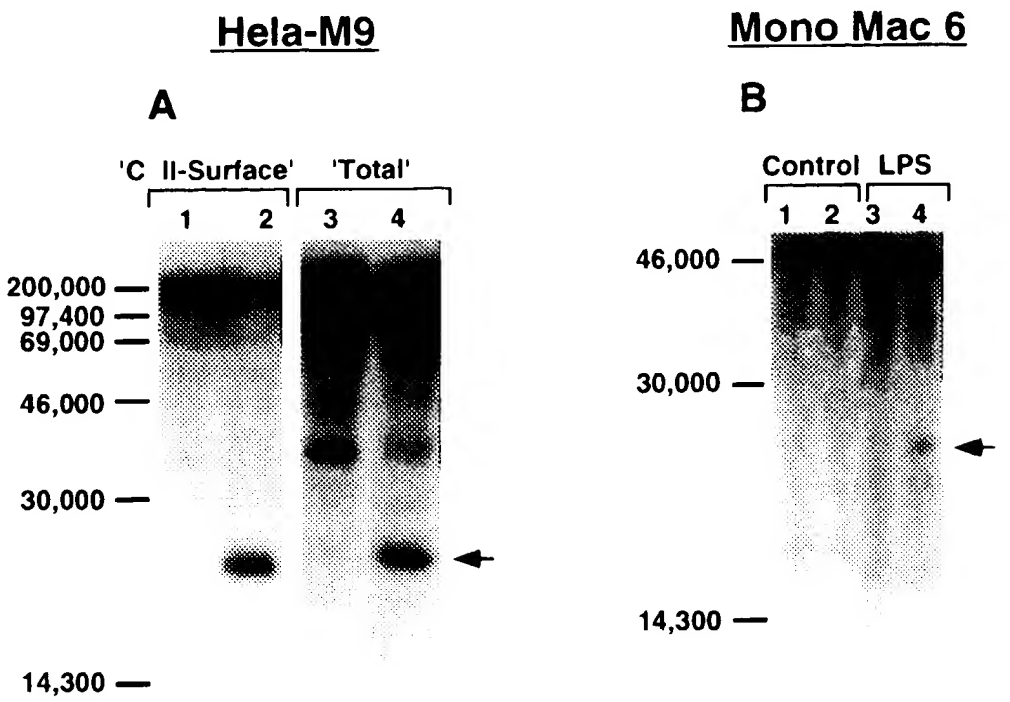

FIGURE 3

Phosphorylation of 26 kDa TNF molecules in HeLa-M9 and LPS-treated MM6 cells. [^{32}P] labeled proteins, immunoprecipitated from lysates of cells that had been metabolically labeled with [^{32}P] orthophosphate with anti-TNF (lanes 2, 4) or control (lanes 1, 3) antibodies. A: Proteins immunoprecipitated from the lysate of HeLa-M9 cells. Immunoprecipitation was performed by adding antibodies either before cell lysis, followed by removal of nonbound antibodies, to specifically detect cell surface TNF molecules (lanes 1, 2: 'cell surface') or after lysis (lanes 3, 4: 'total'). B: Proteins immunoprecipitated from lysates of MM6 cells that had been treated or not treated with LPS (100 ng/ml for 2 hr). The protein samples applied for analysis were from the following number of cells: in A, lanes 1 and 2—1.8×10^6, lanes 3 and 4—0.6×10^6; and in B—2×10^6.

are not phosphorylated. Such analysis could not be performed with 26 kDa TNF molecules from MM6 cells, due to the low amounts of TNF present. However, the mere finding that the 26 kDa molecules are also phosphorylated in these cells is significant; it shows that phosphorylation is not an artifact of the expression of TNF in the HeLa cells, which normally produce little TNF, but rather constitutes part of the normal way of TNF modulation.

The lack of [^{32}P] incorporation in the 17 kDa TNF molecules isolated from the lysate of HeLa-M9 cells indicates that the label in the 26 kDa molecules occurs within their intracellular region. This is to be expected since the intracellular region is the only part of the TNF molecule accessible for phosphorylation by cytoplasmic protein kinases. The specific kinases involved in TNF phosphorylation are not known, nor is it known whether, and in what way, the activity of these kinases is subject to modulation by agents that affect TNF activity. Evidently, the phosphorylation observed in the HeLa-M9 cells, in which TNF was synthesized without stimulation, reflects the function of kinase(s) that constitutively act in these cells. On the other hand, the phosphorylation observed in LPS-stimulated MM6 cells could involve effects of LPS activated protein kinases [21,22]. The serine at position −50 seems to be a suitable substrate for phosphorylation by protein kinase C [23]. However, in preliminary experiments we did not observe any increase of phosphorylation of the 26 kDa TNF molecules in HeLa-M9 cells following treatment with 4β-phorbol-12-myristate-13-acetate (data not shown), suggesting that protein kinase C is either not involved in this phosphorylation or is activated constitutively in these cells, due to their continuous exposure to TNF.

The sequence conservation of the cytoplasmic region of the TNF molecule in different species

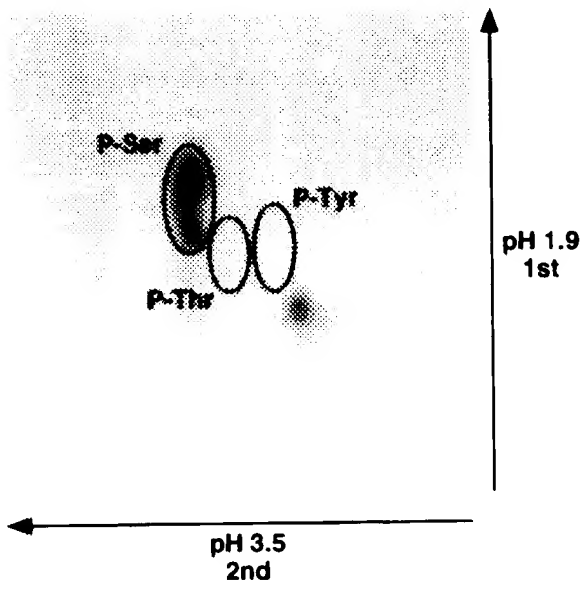

FIGURE 4

Phosphoamino acid analysis of 26 kDa TNF. TNF was immunoprecipitated from the lysate of [^{32}P] orthophosphate labeled HeLa-M9 cells and hydrolyzed, followed by two-dimensional thin layer electrophoretic analysis of the phosphoamino acids. Positions of cold internal phosphoamino acid standards, as determined by ninhydrin staining, are indicated.

indicates that this region and its phosphorylation play important roles in TNF function. Several possible kinds of roles may be considered. One possibility is that this region takes part in the regulation of the proteolytic process by which the soluble 17 kDa form of TNF is derived from the 26 kDa molecule. Involvement of the intracellular region of transmembrane proteins in the regulation of their shedding has been observed for certain proteins. This seems to be the case for the processing of TGF-α which, like TNF, is initially expressed as a transmembrane protein [24], as well as for the induced shedding of the p75 TNF receptor [25]. Contrastingly, shedding of the p55 TNF receptor appears to be independent of the intracellular domain of this receptor [26,27]. The intracellular domain of cell surface TNF may also affect TNF function as a ligand. This region in the molecule may impose conformational changes in the ligand binding of the extracellular TNF domain, or could dictate association with cytoskeletal elements, and thus direct translocation of the TNF molecules within the membrane towards the area of the cell surface adjacent to the target cell. The intracellular region of the Fas ligand, whose structure and activity closely resemble those of TNF, is indeed known to contain a sequence motif, the SH3 binding site, that may allow it to bind to cytoskeletal components [28]. Another possible function of the cytoplasmic region of TNF is interaction with intracellular molecules possessing signaling activities. Activation of signaling activities within the TNF producing cell following the interaction of the cell surface TNF with its target cell may allow fine adjustment of the function or formation of the cell surface TNF molecules, depending on the situation.

Further studies of the phosphorylation of the cytoplasmic TNF domain may contribute not only to our knowledge of the cell surface form of this particular cytokine, but also to our understanding of the mode of action of some other cell surface ligands that are evolutionary related to TNF, including the CD40 ligand (gp39), the OX-40 ligand (the human activation antigen 106, gp34), 4-1BB, and the ligands for CD27, CD30, and for Fas/APO1 [reviewed in ref. 29].

ACKNOWLEDGMENTS

We acknowledge the expert assistance of Dr. Zeev Gechtman in the phosphoamino acid analysis, and the technical assistance of Shlomit Maizel. This study was supported in part by an OTKA grant of the Hungarian Research Foundation (T012915) and by grants from Inter-Lab Ltd., Ness Ziona, Israel, and from Ares Trading S.A., Switzerland.

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TI   A novel domain within the 55 kd   ***TNF***   receptor signals cell
     death.
SO   CELL, (1993 Sep 10) 74 (5) 845-53.
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Cell, Vol. 74, 845–853, September 10, 1993, Copyright © 1993 by Cell Press

A Novel Domain within the 55 kd TNF Receptor Signals Cell Death

Louis A. Tartaglia,* T. M rrill Ayres,*
Grace H. W. Wong,† and David V. Goeddel*
*Department of Molecular Biology
†Department of Cardiovascular Research
Genentech, Inc.
460 Point San Bruno Boulevard
South San Francisco, California 94080

Summary

Deletion mutagenesis of the intracellular region of the 55 kd TNF receptor (TNF-R1) identified an ~80 amino acid domain near the C-terminus responsible for signaling cytotoxicity. This domain shows weak homology with the intracellular domain of Fas antigen, a transmembrane polypeptide that can also initiate a signal for cytotoxicity. Alanine-scanning mutagenesis of TNF-R1 confirmed that many of the amino acids conserved with Fas antigen are critical for the cytotoxic signal. This region of TNF-R1–Fas homology is therefore likely to define a novel domain (death domain) that signals programed cell death. Mutations within the death domain of TNF-R1 also disrupted its ability to signal anti-viral activity and nitric oxide (NO) synthase induction. In addition, large deletions in the membrane-proximal half of the intracellular domain did not block signaling of cytotoxicity or anti-viral activity but did block induction of NO synthase.

Introduction

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages. Although originally identified for its anti-tumor activity, TNF is now known to be one of the most pleiotropic cytokines, signaling a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of many genes (Goeddel et al., 1986; Beutler and Cerami, 1988; Old, 1988; Fiers, 1991). The first step in the induction of the various cellular responses mediated by TNF is its binding to specific cell surface receptors. Two distinct TNF receptors of ~55 kd (TNF-R1) and 75 kd (TNF-R2) have been identified (Hohmann et al., 1990; Brockhaus et al., 1990), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Lewis et al., 1991; Goodwin et al., 1991).

The extracellular domains (ECDs) of human TNF-R1 and TNF-R2 share 28% sequence identity, approximately the same level of similarity they share with the ECDs of a number of diverse cell surface proteins, including the low affinity nerve growth factor (NGF) receptor, Fas antigen, CD40, OX40, and CD27 (Itoh et al., 1991; Camerini et al., 1991). Much of this sequence identity is a result of the extremely well conserved positions of cysteine residues that define this expanding receptor family. There is a complete absence of homology between the intracellular domains of the two TNF receptors, suggesting that they utilize distinct signaling pathways (Lewis et al., 1991). Also, with an exception noted below, the intracellular domains of the two TNF receptors do not show homology to other known proteins (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Lewis et al., 1991).

Numerous studies with anti-TNF receptor antibodies have demonstrated that TNF-R1 is the receptor that signals the large majority of the pleiotropic activities of TNF, including cytotoxicity, fibroblast proliferation, resistance to chlamidiae, synthesis of prostaglandin E_2, anti-viral activity, and manganese superoxide dismutase induction (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992). Transfection-based assays for TNF-R1 have recently been developed by several groups (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992; Wiegmann et al., 1992), confirming the role of TNF-R1 in signaling cytotoxicity, anti-viral activity, and the stimulation of several second messenger pathways. Mutant TNF-R1s in which the majority of the intracellular domain has been removed are defective in initiating cytotoxicity, demonstrating the importance of this domain in mediating TNF signals (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992).

It has recently been shown that the Fas antigen, a member of the TNF/NGF receptor superfamily, can signal a programed cell death very similar to that mediated by TNF (Itoh et al., 1991). Fas antigen is involved in the negative selection of autoreactive T cells, and mice carrying a mutation in the intracellular domain of Fas antigen suffer from a dramatic autoimmune disorder (Watanabe-Fukunaga et al., 1992). In addition, a region of weak homology has been noted between the intracellular domains of TNF-R1 and Fas antigen (Itoh et al., 1991).

While the mechanism of programed cell death is not well understood, the importance of this process in biology is becoming increasingly apparent. The TNF-R1 transfection assay provides an ideal system for defining a signaling domain that can initiate programed cell death. Here we have defined an ~80 amino acid domain within TNF-R1 that can transmit a cytotoxic signal and that is also important in the signaling of other TNF activities. Furthermore, the spacing of this "death domain" relative to the ECD of TNF-R1 can be altered without loss of function.

Results

Delineating the TNF-R1 Death Domain

We showed previously that murine L929 cells expressing a transfected human TNF-R1 could be stimulated by agonist anti-human TNF-R1 antibodies (anti-hR1) to initiate a signal for cytotoxicity (Tartaglia and Goeddel, 1992b). Furthermore, a mutant TNF-R1 lacking the majority of its intracellular domain was defective in signal generation (Tartaglia and Goeddel, 1992b). To define better the se-

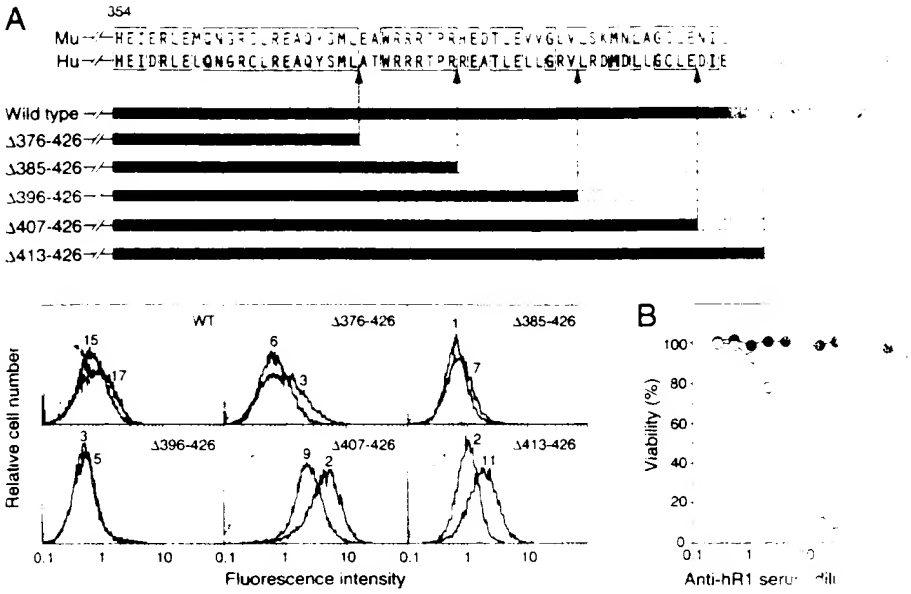

Figure 1. C-Terminal Truncations of TNF-R1

(A) Amino acid sequences of the C-terminal region of murine and human TNF-R1 (Lewis et al., 1991). in the two species. Horizontal bars represent the C-terminus of mutant receptors, shaded regions indicate deleted sequences. Expression of the mutant receptors in individual L929 clones was asse the expression profiles and clone numbers of the two individual L929 clones assayed in Table 1 (dotted curve). Cells were stained with anti-hTNF-R1 MAb 984 and phosphatidylethanolamine-co thousand cells were analyzed per sample. Histograms show relative cell number (y axis) vers axis).

(B) Killing of L929 clones expressing TNF-R1s truncated for 14 and 20 C-terminal residues. Clones (closed circles) were treated with the indicated concentrations of anti-hR1 and 10 μg/ml cyclohe described previously (Tartaglia and Goeddel, 1992b).

quences important for signaling cytotoxicity, we constructed expression vectors for a series of mutant receptors containing various C-terminal truncations (Figure 1A). The mutant receptor constructs were transfected into murine L929 cells, and several independent L929 clones expressing each of the mutant receptors were identified by flow cytometry (Figure 1A). At least two L929 clones expressing each of the mutant receptors were then assayed for a cytotoxic reaction in response to anti-hR1.

Mutant receptors in which 20 or more amino acids had been removed from the C-terminal end were found to be defective in signaling cytotoxicity, while a receptor truncated for the C-terminal 14 amino acids was still functional (Figure 1A and Table 1). The absolute importance of information contained between positions −14 and −20 is further illustrated in Figure 1B, in which the anti-hR1 sensitivities of L929 clones expressing the corresponding receptor mutants are compared in a dose response assay. These data indicate that the C-terminal extension of the cytotoxicity-signaling domain lies between positions −20 and −14.

To define the N-terminal extension of the cytotoxicity signaling domain, a series of TNF-R1 internal deletions were made and expressed in L929 cells. Several of these deletions removed large amounts of sequence in the membrane-proximal half of the intracellular domain without destroying the ability of TNF-R1 to signal cytotoxicity (Figure 2 and Table 1). The largest deletion that did not eliminate th cytotoxic signal extended from amino acid 212 (very close to the transm deletion from amino the ability of TNF-R argue that the N-te signaling domain lie that the C-terminal domain contain suffi ing and cytotoxicity

Homology to Fas

It has been noted p of TNF-R1 shares 45 amino acids) wit gen (Itoh et al., 19 sequences, we not gap in the Fas seq an additional 20 a extensive, this pu TNF-R1 and Fas reaction (Itoh et al ogy falls within the signaling domain the C-terminal lin the 6 amino ac of the cytotoxic

To further val homology and a TNF-R1 that are

Table 1. Signaling of Cytotoxicity by L929 Clones Expr
Human TNF-R1 Deletion Mutants

L929 Clone	Viability (%)
neo.3	98 ± 1
hR1.17	4 ± 1
hR1.15	3 ± 1
Δ376-426.3	100 ± 5
Δ376-426.6	102 ± 1
Δ385-426.1	97 ± 2
Δ385-426.7	96 ± 3
Δ396-426.3	101 ± 2
Δ396-426.5	90 ± 2
Δ407-426.9	94 ± 6
Δ407-426.20	101 ± 4
Δ413-426.11	2 ± 1
Δ413-426.2	10 ± 2
Δ258-308.16	17 ± 2
Δ258-308.19	12 ± 2
Δ212-308.2	7 ± 2
Δ212-308.29	4 ± 3
Δ258-326.9	15 ± 8
Δ258-326.24	45 ± 8
Δ212-326.2	55 ± 7
Δ212-326.5	43 ± 8
Δ212-340.20	98 ± 8
Δ212-340.21	101 ± 4

Two independent L929 clones were examined for each TNF-R1 dele-
tion mutant. Cells were treated for 24 hr with a 1:400 dilution of aga
anti-human TNF-R1 antibody (anti-hR1) in the presence of 10 u
cycloheximide. Values are expressed as percentage viability (±
n = 3) compared with the same cells treated with cycloheximide alo
L929 clone neo.3 is a G418-resistant control. hR1.17 and hR1.15 ex-
press the wild-type human TNF-R1 and have been referred to pre-
viously as L929.hR1.17 and L929.hR1.15 (Tartaglia and Goeddel,
1992b).

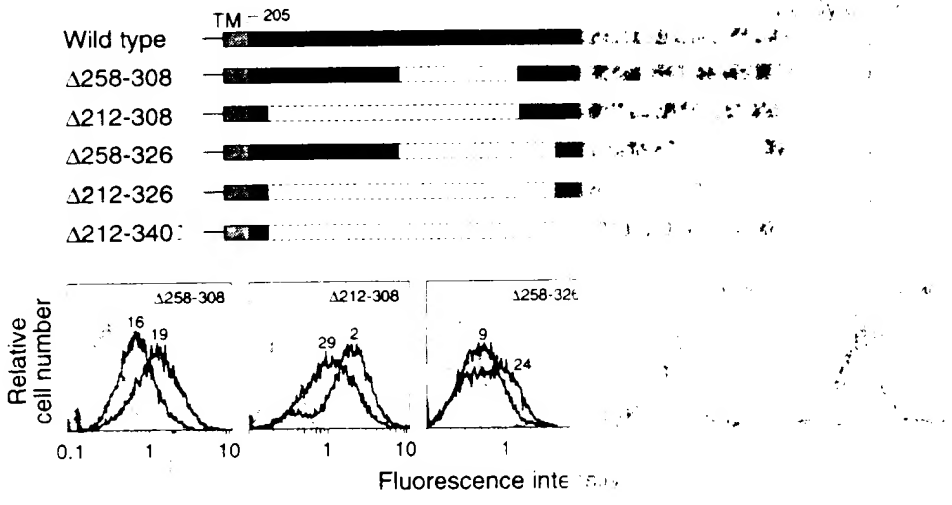

Figure 2. Internal Deletions within the TNF-R1 Intracellular Doma

The locations of deleted intracellular domain sequence in four mut
sequence; dotted regions indicate deleted sequences. Expression c
analysis as in Figure 1. Shown are the expression profiles and c
with a L929-neo control clone (dotted curve).

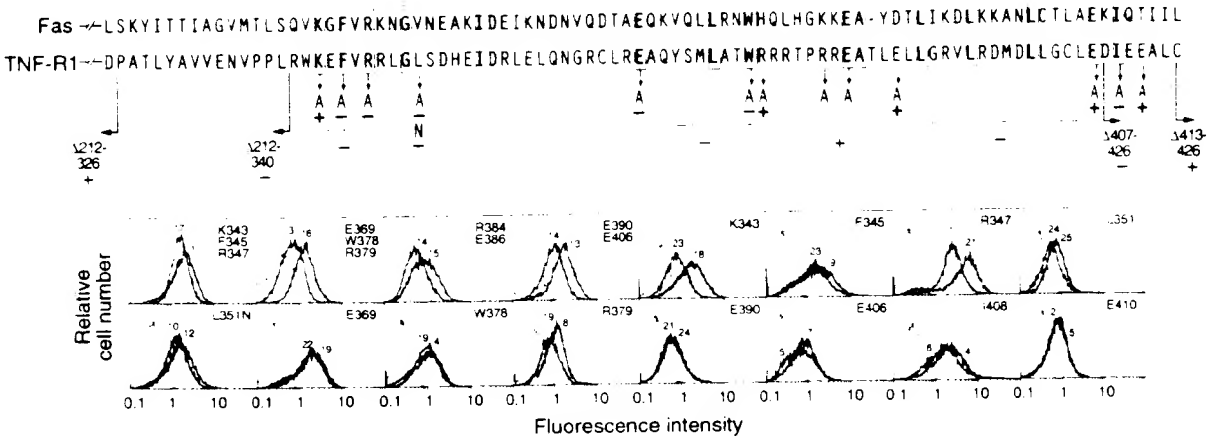

Figure 3. Substitution Mutagenesis of the Death Domain

The amino acid sequence of the TNF-R1 death domain (amino acids 326–413) is compared with the homologous region in the Fas antigen intracellular domain. Amino acids identical in the two sequences are stippled. The N-terminal and C-terminal deletions defining the death domain are indicated. Amino acids converted to alanine (A) by site-directed mutagenesis are indicated by an arrow pointing to the letter A. Leu-351 was also changed to Asparagine (N). A minus sign immediately below the letter A or N indicates that TNF-R1 could not signal cytotoxicity when this single amino acid replacement was made. A minus sign immediately below a bracket indicates that TNF-R1 could not signal cytotoxicity when the corresponding group of 2 or 3 amino acids was mutagenized. Expression of the mutant receptors in individual L929 clones was assessed by flow cytometric analysis as in Figure 1. Shown are the expression profiles of the individual L929 clones assayed in Table 2 in comparison with a L929-neo control clone (dotted curve)

65 amino acid homology region (from Phe-345 to Ile-408). This may suggest that a folded protein domain within TNF-R1 makes multiple noncontiguous contacts with other proteins involved in signal transduction. Third, the critical information between positions −14 and −20 (identified in the deletion analysis) may be the Ile that is conserved between Fas and TNF-R1, since substitution of only this conserved Ile results in a defective cytotoxic signal.

To determine whether the region within TNF-R1 that is responsible for signaling cytotoxicity is interchangeable with the corresponding region in the Fas antigen, a TNF-R1–Fas antigen fusion was generated and expressed in L929 cells. The fusion protein contains TNF-R1 sequences from position 1 to 323 fused to Fas sequences from position 210 to 319. This fusion therefore replaces TNF-R1 sequences beginning 3 amino acids before the N-terminal extension of the death domain with the corresponding Fas antigen sequences. The position of this fusion junction also results in a precise swap of amino acids encoded on the final exons of the TNF-R1 and Fas antigen genes (Fuchs et al., 1992; S. Nagata, personal communication). Three independently isolated L929 clones expressing this fusion protein showed modest cytotoxicity in response to anti-hR1 (Table 2), further demonstrating the relevance of the homology between these two proteins.

Positional Flexibility of Death Domain Relative to ECD

Considerable evidence has accumulated that the mechanism of TNF-R1 triggering involves the cross-linking of receptors by the TNF ligand (reviewed by Tartaglia and Goeddel, 1992a). It has also been demonstrated that the association of intracellular domains is critical in signal generation (Tartaglia and Goeddel, 1992b). It therefore surprised us when large internal deletions between the transmembrane region and the death domain did not interfere with signaling. These internal deletions might be expected to destroy the register between the ligand cross-linked ECDs and the associating intracellular domain sequences. Therefore, information contained in the orientation of TNF-R1 cross-linking would be lost upon removal of internal sequences and could potentially interfere with proper association of the intracellular domains. However, a possible explanation for the positional flexibility of the death domain relative to the ECD in our experiments is that polyclonal antibodies were used as ligand. These antibodies may cross-link the TNF-R1 molecules in a variety of orientations. We tested whether TNF itself could trigger cytotoxicity through mutant receptors with altered spacing between the ECDs and the death domain. This required blocking of the endogenous murine TNF-R1 on the L929 clones by pretreatment with an antagonist monoclonal antibody (MAb) against murine TNF-R1. These cells were then treated with TNF (which now has access only to the transfected human TNF-R1) and assayed for cytotoxicity. L929 cells expressing either the wild-type human TNF-R1 or mutant TNF-R1s with large deletions between the ECD and the death domain were all sensitive to TNF, even after access to the endogenous murine TNF-R1 was blocked (Figure 4). This indicates that the death domain has positional flexibility relative to the TNF cross-linked ECDs and suggests that information contained in the orientation of receptor aggregation may not be critical in signal generation.

Effect of TNF-R1 Mutations on the Signaling of Antiviral Activity and the Induction of Nitric Oxide Synthase

TNF-R1 is known to signal a large number of diverse biological activities in addition to cytotoxicity. We thus were

Table 2 Signaling of Cytotoxicity by Human TNF-R1 Mutants

L929 Clone	Viability (%)
neo.3	95 ± 2
hR1.17	4 ± 1
hR1.15	3 ± 1
K343, F345, R347.1	99 ± 5
K343, F345, R347.17	102 ± 3
E369, W378, R379.3	92 ± 4
E369, W378, R379.16	96 ± 5
R384, E386.14	3 ± 1
R384, E386.15	30 ± 5
E390, E406.13	97 ± 6
E390, E406.14	96 ± 3
K343.18	22 ± 5
K343.23	43 ± 2
F345.9	90 ± 2
F345.23	87 ± 16
R347.1	88 ± 4
R347.21	94 ± 7
L351.24	109 ± 5
L351.25	97 ± 2
L351.10→N	101 ± 8
L351.12→N	97 ± 4
E369.19	80 ± 7
E369.22	93 ± 2
W378.19	92 ± 8
W378.4	88 ± 6
R379.8	16 ± 3
R379.19	2 ± 2
E390.21	75 ± 4
E390.24	47 ± 5
E406.5	30 ± 1
E406.7	50 ± 10
I408.4	86 ± 3
I408.6	87 ± 9
E410.2	21 ± 2
E410.5	2 ± 1
R1–Fas.3	59 ± 9
R1–Fas.9	68 ± 7

Two independent L929 clones were examined for each TNF-R1 mutant. Cells were treated for 24 hr with a 1:400 dilution of anti-hR1 polyclonal antibody in the presence of 10 μg/ml cycloheximide. Values are expressed as percentage viability (± SD, n = 3) compared with the same cells treated with cycloheximide alone. All listed mutations were alanine substitutions unless otherwise indicated.

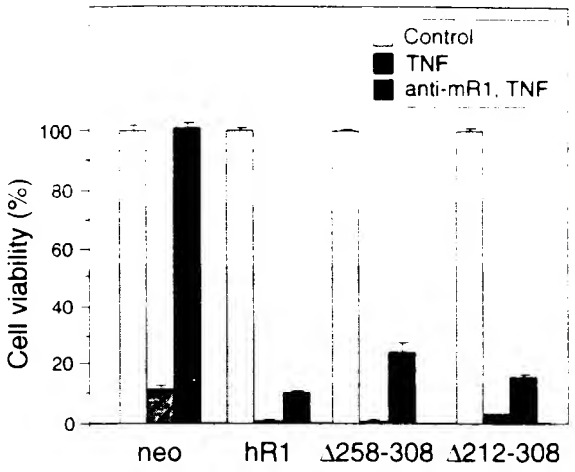

Figure 4. TNF Killing of L929 Cells Expressing TNF-R1s with Altered Spacing between the ECD and Death Domain

A control L929 clone (neo.5) and L929 clones expressing wild-type (hR1.17) or mutant TNF-R1s (Δ258-308.16 and Δ212-308.2) were pretreated for 1 hr with a 1:10 dilution (hybridoma supernatant) of anti-murine TNF-R1 MAb 176. Cells were then further treated with 100 ng/ml TNF for 24 hr in the absence of cycloheximide. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992b).

interested to learn whether mutations that affected the signaling of cytotoxicity altered the signaling of other TNF activities. We have recently shown that the known anti-viral activities of TNF are signaled by TNF-R1, and that human TNF-R1 (together with interferon γ [IFN-γ]) can transmit an anti-viral signal when expressed in murine L929 cells (Wong et al., 1992). We therefore assayed mutant human TNF-R1s expressed in L929 cells for their ability to transmit a signal that results in protection from subsequent infection by vesicular stomatitis virus (Table 3). All mutations tested within the death domain that blocked the signaling of cytotoxicity also eliminated the signaling of anti-viral activity, while the mutations that did not eliminate the cytotoxic signal also did not interfere with the anti-viral signal. In addition, the large deletions between the ECD and death domain that did not block the signaling of cytotoxicity also did not block the signaling of anti-viral activity. This inability to separate th signaling of cytotoxicity and anti-viral activity by the many TNF-R1 mutations suggests that a common signal from TNF-R1 initiates both the cytotoxic and anti-viral programs.

Another important biological activity mediated by TNF in combination with IFN-γ is the induction of a nitric oxide (NO) synthase activity (Farrar et al., 1992). Preliminary experiments with agonist antibodies to murine TNF-R1 and TNF-R2 showed that in L929 cells this TNF activity was mediated by murine TNF-R1 (data not shown). In addition, human TNF-R1 could initiate NO induction in L929 cells that were transfected with wild-type human TNF-R1 (see below). To determine whether the same mutations that interfere with the signaling of cytotoxicity and anti-viral activity also interfere with the induction of NO synthase, we assayed L929 clones expressing the mutant human TNF-R1s for NO synthase induction in response to anti-hR1 and murine IFN-γ (mIFN-γ). As shown in Table 3, mutations within the death domain of TNF-R1 that are negative for cytotoxicity are also negative for NO synthase induction. The negative data obtained for these mutant receptors are not due to the inability of the host L929 clones to induce NO synthase, because these clones could still induce NO through the endogenous murine TNF-R1 in response to either anti-murine TNF-R1 antibodies or TNF itself (data not shown). Mutations within the death domain that did not block the signaling of cytotoxicity also did not block the signaling of NO synthase induction. Therefore, the signal initiated from the death domain that is responsible for cytotoxicity may also be required for NO synthase induction. Interestingly, all of the large deletions between the transmembrane domain and the death domain eliminated the signaling of NO synthase induction (Table 3). The signaling of NO synthase induction thus appears to require both an intact death domain and additional sequences in the N-terminal half of the intracellular domain.

Table 3. Summary of TNF-R1 Mutational Analysis

Receptor Mutation	Signaling: Cytotoxicity	Signaling: Antiviral	Signaling: NO Synthase Induction
Wild type	+	+	+
Δ245-426	–	–	–
Δ376-426	–	ND	ND
Δ385-426	–	ND	ND
Δ396-426	–	ND	ND
Δ407-426	–	–	–
Δ413-426	+	+	+
K343, F345, R347→A	–	–	–
K343→A	+	+	+
F345→A	–	–	–
R347→A	–	–	–
L351→A	–	–	–
L351→N (lpr)	–	–	–
E349, W378, R379→A	–	ND	–
E369→A	–	–	–
W378→A	–	–	–
R379→A	+	+	+
R384, E386→A	+	+	+
E390, E406→A	–	–	–
E390→A	+/–	+	+
E406→A	+	+	+
I408→A	–	–	–
E410→A	+	+	+
Δ258-308	+	+	–
Δ258-326	+	+	–
Δ212-308	+	+	–
Δ212-326	+	+	–
Δ212-340	–	–	ND

For each of the indicated mutations, at least two independently isolated L929 clones expressing the corresponding mutation were analyzed. Triplicate determinations were made on each L929 clone for the cytotoxicity (see Tables 1 and 2) and NO assays, and sextuplicate determinations were made for the antiviral assay. A plus sign in the cytotoxicity assay indicates greater than 50% cytotoxicity under the conditions described in Tables 1 and 2, while a minus sign indicates less than 20% cytotoxicity. A plus sign in the antiviral assay indicates greater than 75% survival under the conditions described in Experimental Procedures, while a minus sign indicates less than 25% survival. In the antiviral assay, agonist antibody activation of the wild-type receptor in hR1.17 cells resulted in 80% ± 2% survival. Survival in untreated cells was typically between 15% and 20%. The reliable detection limit of the NO synthase assay was 0.3 nmol of nitrite per 10^5 cells under the conditions described in Experimental Procedures. A minus sign indicates that nitrite levels measured after induction were below the reliable detection limit. A plus sign indicates that nitrite levels were induced to greater than 0.8 nmol per 10^5 cells. In the NO assay, agonist antibody activation of the wild-type receptor in hR1.17 cells resulted in a measured nitrite level of 2.6 ± 0.2 nmol per 10^5 cells. ND, not determined.

A Subset of TNF-R1 Negative Mutations Have Dominant Negative Character

In a previous report, we demonstrated that expression in L929 cells of a truncated human TNF-R1 (missing the majority of its intracellular domain) resulted in suppressed signaling by the endogenous murine TNF-R1 (Tartaglia and Goeddel, 1992b). This dominant negative effect was due to TNF cross-linking the functional endogenous receptors to the nonfunctional truncated receptors. The resulting receptor complexes lacked interacting intracellular domains and were therefore defective in signal generation (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992). As described above, we have now identified a number of negative mutations within human TNF-R1 that result from only minor changes in intracellular domain sequence. We were now interested whether these less extensive mutations would also act as dominant negative mutations.

As revealed by previous work (Tartaglia and Goeddel, 1992b) and our preliminary experiments, an assessment of the dominant negative character of a mutant transmembrane receptor is complicated by two factors: first, the extent of the dominant negative effect is highly dependent on the expression level of the mutant receptor; and second, even mutant receptors that do not have true dominant negative character can appear to reduce the sensitivity of a cell to TNF in a dose response assay if the ligand is titrated from the assay media. To circumvent the first problem, we restricted our analysis to only those few cell lines in which the expression level of human TNF-R1 was equal to or greater than that in a well-characterized control cell line (L929.hR1Δ.4; Tartaglia and Goeddel, 1992b). This control line expresses a truncated TNF-R1 at a level that significantly suppresses signaling by the endogenous murine TNF-R1. To circumvent the problem of ligand titration from the assay media, we examined the TNF sensitivity of L929 clones under conditions of extreme ligand excess (50 nM) and in the absence of cycloheximide. Several of the TNF-R1 mutations clearly acted as dominant negative mutations, as evidenced by the decreased TNF sensitivity of the corresponding L929 clones (Table 4). As shown previously for the L929.hR1Δ.16 clone (Tartaglia and Goeddel, 1992b), the decreased sensitivity of these clones was not due to differences in murine TNF-R1 levels or the signal transduction apparatus, because pretreatment with a human TNF-R1 antagonist antibody restored normal sensitivity (data not shown). Interestingly, several of the cytotoxicity-signaling negative mutations did not act as dominant negative mutations, despite high level receptor expression. There was a clear pattern in the intracellular domain location of these mutations. Mutations that disrupted the N-terminal half of the death domain had dominant negative character, while those that disrupted information in the C-terminal half of the death domain did not.

Discussion

Despite the absence of recognizable signaling motifs or kinase homologies, the 55 kd TNF receptor (TNF-R1) signals a large number of diverse biological activities. Of particular interest is its ability to initiate a rapid cytotoxic program, since the signaling mechanisms of programed cell death are poorly understood. To understand better the mechanisms by which TNF-R1 signals cell death and other important biological responses, we have begun to define sequences within its intracellular domain that are required for function.

Through a series of both C-terminal truncations and internal deletions, we have identified an ~80 amino acid domain within the 221 amino acid intracellular region of TNF-R1 that is required and sufficient for initiating the signal for cytotoxicity. The C-terminal extension of this domain is close to the C-terminus of the receptor (removal

Table 4. Dominant Negative Character of Human TNF-R1

L929 Clone	Viability (%)
neo.3	1.7 ± 0.5
Δ245-426.4	55.7 ± 2.3
Δ212-340.8	78.9 ± 4.7
K343, F345, R347.1	21.2 ± 1.0
F345.9	33.4 ± 1.0
R347.1	35.4 ± 0.5
R347.21	50.3 ± 2.2
E390, E406.13	5.7 ± 2.1
I408.4	6.4 ± 0.5
Δ407-426.2	1.8 ± 0.6
Δ407-426.9	2.0 ± 0.4

Cells were treated for 18 hr with 50 nM TNF. Values are expres as percentage viability (± SD, n = 6) compared with controls. Cl Δ245-426.4 was referred to previously as L929.hR1Δ.4 (Tartaglia Goeddel, 1992b).

of the terminal 20 but not the terminal 14 amino acic eliminates signaling), and the N-terminal extension is ne the center of the intracellular domain primary sequen Sequence information within the N-terminal half of the i tracellular domain does not appear to be required for the cytotoxic signal. The results of both the C- and N-termin deletion analysis are very consistent with the homolog between the mouse and human TNF-R1s (Lewis et al 1991). Strong conservation between the murine and hu man TNF-R1s begins very close to the N-terminal extension of the death domain. In addition, this homology drops off abruptly in the C-terminal 14 amino acids.

The 80 amino acid cytotoxicity-signaling domain (death domain) within TNF-R1 contains a region of 65 amino acids that shows 28% identity to a region within the intracellular domain of Fas antigen. Although this homology is not extensive, the Fas antigen can signal a programed cell death very similar to that signaled by TNF-R1 (Itoh et al., 1991), thus establishing a functional conservation between the two receptors. To test the relevance of the amino acid sequence homology, we performed alanine scanning mutagenesis on amino acids within the intracellular domain of TNF-R1 that are conserved with the Fas antigen. This revealed a number of residues within TNF-R1 that are critical for TNF-R1 function, further validating the significance of the homology between the intracellular domains of these two receptors. A number of these essential residues are charged and so are likely to be exposed on the surface of the death domain. These residues may thus represent positions at which the TNF-R1 intracellular domain interacts with cytoplasmic proteins or possibly other TNF-R1 intracellular domains. The large number of such essential residues scattered throughout a region of ~65 amino acids suggests that TNF-R1 does not display a short peptide that binds and stimulates an intracellular signaling component, but rather makes multiple noncontiguous contacts with a folded structural domain. However, our experiments do not rul out the possibility that some of the amino acid replacements may disrupt proper folding of the intra cellular domain. Wheth r Fas and TNF-R1 interact wit common or different intracellular signaling proteins re mains to be determined. It is possible that the relative

yama et al., 1992). The interleukin 2 β chain requires only its serine-rich domain to signal for proliferation in BAF3 cells, yet c-*fos* induction requires both the serine-rich domain and an additional region.

The triggering of TNF-R1 is a consequence of the cross-linking of TNF-R1 monomers by either anti-receptor antibodies or the TNF trimer (reviewed by Tartaglia and Goeddel, 1992a). The analysis discussed above has defined amino acids that, when mutated in all chains within a homoreceptor complex, destroy the ability to function. However, we were also interested in distinguishing between intracellular domain information that is required in all TNF-R1 molecules and information that is more redundant in the receptor complex. An analysis of the dominant negative character of a mutant transmembrane receptor should provide insight into this. Those receptors mutated for intracellular domain information that is required in all receptor chains within a complex should suppress signaling when cross-linked to functional receptors. Alternatively, mutations in information not required in all receptor chains would not be expected to have strong dominant negative character upon their cross-linking to intact receptors.

Our analysis of several inactive TNF-R1s identified both classes of mutations: those that had dominant negative character and those that did not. The differences observed between these two classes were not a consequence of expression level, but rather were a function of the location at which the mutation occurred in the intracellular domain. Those mutations that disrupted information in the N-terminal half of the death domain had dominant negative character, while those that disrupted information in the C-terminal half did not. One possible explanation for these two phenotypes is that sequences in the N-terminal half of the death domain are involved in intracellular domain association. Therefore, when the TNF ligand cross-links such mutant receptors to wild-type receptors, proper intracellular domain association does not occur, and the receptor complex cannot be activated for signaling. In contrast, sequences in the C-terminal half may not be critical for intracellular domain association and the subsequent conversion to an active complex; rather, they may associate with intracellular signaling molecules, and the association of every receptor chain with a signaling molecule may not be essential for the generation of a signal.

A more complete understanding of the signaling of programed cell death will require both structural studies on the death domains of TNF-R1, Fas antigen, and other similar molecules and the identification of intracellular molecules that interact with them. It will also be of interest to learn whether there exists a family of intracellular killing proteins that provides the specificity for programed cell death in different tissues. This information might provide the tools to manipulate cell death both positively and negatively for the treatment of many disease states.

Experimental Procedures

Reagents

Recombinant human TNF (of specific activity over 10^7 U/mg) and mIFN-γ were provided by the Genentech manufacturing group. The rabbit anti-murine TNF-R1 and rabbit anti-hR1 polyclonal agonist antibodies have been described previously (Tartaglia and Goeddel, 1991; Tartaglia and Goeddel, 1992b). The titer of the anti-hR1 was 1:150,000, as quantitated by a direct antigen-coated enzyme-linked immunosorbent assay. MAb 984 against human TNF-R1 inhibits the binding of TNF to human TNF-R1 and has also been described previously (Tartaglia and Goeddel, 1992b). MAb 176 against murine TNF-R1 inhibits the binding of TNF to murine TNF-R1 (Tartaglia et al., 1993).

TNF-R1 Mutagenesis

The starting plasmid for the TNF-R1 mutagenesis contained the cDNA encoding wild-type TNF-R1 cloned into the Rous sarcoma virus long terminal repeat expression vector pRIS and has been described previously (Tartaglia and Goeddel, 1992b). Plasmids encoding human TNF-R1s with C-terminal truncations were generated by replacement of sequences between the HindIII restriction site and convenient restriction sites with synthetic DNA containing an in-frame stop codon. Verification of correctly modified cDNAs was determined by double-strand DNA sequencing. All internal deletions and substitution mutations were generated by site-directed mutagenesis as follows. Regions of the TNF-R1 to be mutated were subcloned into Bluescript SK(+) (Stratagene) and made single stranded in the *dut⁻ ung⁻* F' Escherichia coli CJ236 strain. Oligonucleotides that contained the desired mutation and were complementary to the single-stranded template were used for primer extension (Kunkel et al., 1987). The primer-extended product was transformed into DH5αF'. Following DNA sequencing to confirm the sequence of the mutated region, the TNF-R1 fragment was subcloned back into the pRIS expression vector.

Generation of Murine L929 Clones Expressing Human TNF-R1

The expression vectors encoding the mutant human TNF-R1s were introduced into mouse L929 cells by electroporation. Cells (5×10^6 in 1.0 ml) were cotransfected with 0.5 μg of ScaI-digested pRK.neo and 20 μg of ScaI-digested TNF-R1 expression vector. Cells were plated into 15 cm plates and, after 2 days, selected in medium containing 600 μg/ml G418. After 12 days, individual G418-resistant clones were picked and expanded. To examine the expression of human TNF-R1, cells were incubated on ice for 60 min with 100 μg/ml anti-hR1 MAb 984 in phosphate-buffered saline containing 2% fetal bovine serum. The cells were then washed and stained with phosphatidylethanolamine-conjugated goat anti-mouse immunoglobulins (Caltag Laboratories) and analyzed on an Epics Elite instrument (Coulter Electronics).

L929 Cytotoxicity Assay

L929 cells (2×10^4 per well) were seeded into 96-well microtiter plates in 100 μl of medium (low glucose Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin; GIBCO) and incubated for 24 hr at 37°C in a 5% CO_2 atmosphere. The medium was then brought to 10 μg/ml cycloheximide, and the anti-hR1 or TNF was added to the wells and serially diluted. The plates were incubated for an additional 24 hr (or 18 hr for the dominant negative assay) and the viable cells stained with 20% methanol containing 0.5% crystal violet. The dye was eluted with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol, and absorbance was measured at 540 nm.

NO Assay

Cells were seeded at 1×10^5 cells/ml in Corning 24-well tissue culture plates, incubated at 37°C for 24 hr, and then treated with mIFN-γ (50 U/ml), alone or in combination with TNF (100 ng/ml), anti-hR1 (1:400 dilution), or anti-mR1 (1:1000 dilution). After 48 hr at 37°C, supernatants were assayed for nitrite by the Greiss reaction (Green et al., 1982).

Antiviral Assay

A suspension of cells (100 μl) at 2×10^5/ml in DMEM supplemented with a 5% fetal calf serum was added to each well of a 96-well plate for 24 hr before the assay. Anti-hR1 was then added to the attached cells at a 1:400 dilution in combination with 0.1 ng/ml mIFN-γ. After 24 hr, cells were challenged with vesicular stomatitus virus diluted in DMEM with 2% fetal calf serum at a multiplicity of infection of 0.1 and were further incubated at 37°C. After 24 hr, virus control wells were checked by microscopic examination to confirm >80% lysis. The fluid

from all wells was poured off, and the attached viable cells were stained with 0.5% crystal violet in 20% methanol for 15 min at ambient temperature. Cell viability was determined by eluting the dye from the stained cells with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol and measuring absorption at 540 nm. No anti-viral activity was mediated by mIFN-γ alone under the conditions of this assay.

Acknowledgments

We would like to thank Barbara Stallard for construction of the Δ376-426, Δ385-426, and Δ396-426 mutant TNF-R1 cDNAs. We also thank Kerrie Andow and Wayne Anstine for computer graphics work and Sunita Sohrabji for manuscript formatting.

Received April 14, 1993; revised July 1, 1993.

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AU   Song H Y; Dunbar J D; Donner D B
TI   Aggregation of the   ***intracellular***     ***domain***   of the
     type 1   ***tumor***     ***necrosis***     ***factor***   receptor
     defined by the two-hybrid system.
SO   JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 Sep 9) 269 (36) 22492-5.
     Journal code: HIV. ISSN: 0021-9258.
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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 36, Issue of September 9, pp. 22492–22495, 1994

Aggregation of the Intracellular Domain of the Type 1 Tumor Necrosis Factor Receptor Defined by the Two-hybrid System*

(Received for publication, June 8, 1994, and in revised form, July 7, 1994)

Ho Yeong Song, James D. Dunbar, and David B. Donner‡

From the Department of Physiology and Biophysics and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202

The yeast-based two hybrid system has been used to determine whether oligomerization of the intracellular domain of the 55-kDa type 1 tumor necrosis factor (TNF) receptor may occur during TNF action. This assay depends upon reconstitution of the function of the GAL4 transcriptional activator through interaction of a protein fused to the GAL4 DNA binding domain with a protein fused to the transcriptional activation domain of GAL4. Fusion of the type 1 TNF receptor intracellular domain with the DNA binding domain and the transactivation domain of GAL4 led to activation of the *lacZ* indicator gene, demonstrating interaction of the receptor intracellular domain with itself. A HeLa cell cDNA library was searched for proteins that interact with the intracellular domain of the type 1 TNF receptor. A protein corresponding to amino acids 329–426 in the type 1 TNF receptor intracellular domain was identified by this screen. The aggregation domain was further defined by testing the ability of deletion mutants of the type 1 TNF receptor intracellular region to interact with the complete intracellular domain. These experiments map the aggregation domain to a sequence of amino acids previously shown to be responsible for mediating TNF-induced cytotoxicity. These results suggest that aggregation of type 1 TNF receptor intracellular domains may be important in TNF signal transduction.

Tumor necrosis factor (TNF)[1] is a multifunctional cytokine produced predominantly by macrophages activated by infections or malignancies (1–3). TNF induces the hemorrhagic necrosis and regression of cancers in animals by inducing an inflammatory response in tumor capillary beds and elicits apoptosis through direct interactions with transformed cells. TNF also promotes immunity, antiviral responses, inflammation, shock, and, in some chronic diseases, the syndrome of wasting and malnutrition known as cachexia (1–3).

The first step in TNF action is binding to specific receptors that are expressed on virtually all cells (4–7). Two distinct receptors for TNF have been identified and characterized as proteins of 55 kDa (the type 1 receptor, TNFR-1) and 75 kDa (the type 2 receptor, TNFR-2) (8–11), and the cDNAs encoding each have been cloned (12–14). The extracellular domains of the TNF receptors share homologies with one another and with a group of cell surface receptors, which include the FAS antigen, the low affinity NGF receptor, 4–1BB, CD40, OX40, and CD27 (15, 16). The intracellular domains of members of the TNF/NGF receptor superfamily are distinct and are believed to couple to different signal transduction pathways. Consistent with this, the TNF receptors induce different cellular responses. TNFR-1 promotes cytotoxicity, fibroblast proliferation, antiviral responses, and the host defense against microorganisms and pathogenic factors (10, 17–20); TNFR-2 induces the proliferation of T cells (21).

Deletion analysis of the intracellular domain of TNFR-1 (TNFR-1IC) has identified a sequence of about 80 amino acids in the C terminus responsible for inducing cytotoxicity (22). Overexpression of non-functional TNFR-1 deletion mutants lacking portions of the cytoplasmic domain suppress signaling by non-defective, endogenous TNF receptors (23, 24). One interpretation of the ability of the dominant negative mutants to abrogate TNF-induced cytotoxicity is that aggregation of TNFR-1IC is necessary for initiating signal transduction (22, 23).

The present studies were initiated to directly test whether the TNFR-1IC is capable of aggregation. To test this experimentally, we have used the two-hybrid system, a yeast-based method for studying protein-protein interactions (25, 26). The method is based on the properties of the yeast GAL4 protein, which consists of separable domains that mediate DNA binding and transcriptional activation. Plasmids encoding two hybrid proteins, one consisting of the GAL4 DNA binding domain fused to protein X and the other consisting of the GAL4 activation domain fused to protein Y, are co-transformed into yeast. Interaction between proteins X and Y permits transcriptional activation of an integrated copy of the *gal4-lacZ* reporter gene. The yeast two-hybrid system has also been used to screen cDNA expression libraries for genes encoding proteins that interact with any protein under study. In this manifestation of the method, yeast are co-transformed with a plasmid expressing the GAL4 DNA binding domain fused to the protein of interest and a pool of plasmids encoding GAL4 activation domain cDNA library fusion proteins.

To determine whether TNFR-1IC is capable of aggregation, plasmids in which this domain was fused with both the GAL4 DNA binding domain and the GAL4 trans-activation domain were constructed. Co-transformation of these plasmids into yeast activated the *lacZ* indicator gene, indicating interaction of TNFR-1IC with itself. We have also screened a HeLa cell cDNA library for encoded proteins capable of binding to TNFR-1IC. One protein identified by this procedure corresponded to amino acids 329–426 within TNFR-1IC, which must contain the domain essential for aggregation. Finally, we assayed the ability of deletion mutations of TNFR-1IC to interact with the

* This work was supported by a grant from the Indiana Affiliate of the American Diabetes Association (to D. B. D.) and by a postdoctoral fellowship from the Indiana Affiliate of the American Heart Association (to H. Y. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed: Dept. of Physiology and Biophysics and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202. Tel.: 317-278-2155; Fax: 317-274-3318.

[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR-1, 55-kDa tumor necrosis factor receptor; TNFR-2, 75-kDa tumor necrosis factor receptor; TNFR-1IC, intracellular domain of TNFR-1; TNFR-2IC, intracellular domain of TNFR-2; NGF, nerve growth factor.

complete intracellular domain. These experiments map the aggregation domain to the sequence of amino acids in the C terminus of TNFR-1 responsible for mediating cytotoxicity.

MATERIALS AND METHODS

Bacterial and Yeast Strains—All yeast strains and plasmids for two-hybrid experiments were obtained from Clontech (Palo Alto, CA) as components of the MATCHMAKER Two Hybrid System. Yeast strains SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can',gal4-542, gal80-538, URA3::GAL1-lacZ*) and HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ*) were used to assay for protein-protein interactions. Yeast strain HF7c was used for library screening. SFY526 has the upstream activating sequence and TATA sequences of the GAL1 promoter fused to the *lacZ* gene. In HF7c, HIS3 is fused to a GAL1 promoter sequence and LacZ is fused to three copies of a 17-mer GAL4 consensus sequence plus the TATA sequence of the CYC1 promoter. Both HIS3 and LacZ are responsive to GAL4 activation. The *Escherichia coli* strain of XL1-blue (Stratagene) was employed in the cloning of plasmids unless otherwise noted. Transformation of yeast strains were performed according to the instructions in the MATCHMAKER Two Hybrid System.

Construction of pGBT9-55IC and pGAD424-55IC—Yeast shuttle vector plasmids containing the GAL4 DNA binding domain (pGBT9) and the GAL4 activation domain (pGAD424) as well as the control plasmids pCL1 (the wild type full-length *GAL4* gene), pVA3 (the p53 gene), pTD1 (SV40 large T antigen), and pLAM5′ (the lamin gene) were from Clontech. For insertion of TNFR-1IC into fusion vectors, two unique cloning sites in both pGBT9 and pGAD424, *Eco*RI and *Bam*HI, were used. PCR primers were designed to amplify TNFR-1IC using a cDNA clone of TNFR-1 in pUC19 (a gift from Dr. H. Loetscher, Hofmann-LaRoche Inc., Geneva, Switzerland) as the template with the *Eco*RI and *Bam*HI restriction sites linked to the 5′ and 3′ end primers, respectively. TNFR-2IC (a gift from Dr. H. Loetscher) and the human FAS antigen (a gift from Dr. S. Nagata, Osaka Bioscience Institute, Osaka, Japan) intracellular domain were amplified and subcloned into pGBT9 and pGAD424 by a procedure similar to that described for TNFR-1IC. PCR products were run on a low melting point agarose gel, cut out, melted, cleaned using a DNA Cleanup Kit (Promega), digested with appropriate enzymes, and finally ligated to the appropriate vector. Plasmid isolation was accomplished using the Wizard Miniprep and Maxiprep kits from Promega (Madison, WI).

Library Screening—The human HeLa S3 MATCHMAKER cDNA library was purchased from Clontech. pGBT9-TNFR-1IC was transformed into HF7c using the lithium acetate procedure as described in the manufacturer's instructions for the MATCHMAKER Two Hybrid System. Colonies of this transformant were confirmed as His$^-$ and LacZ$^-$ to insure that the TNFR-1IC alone does not contain transcriptional activity in HF7c. The HF7c transformant was grown overnight in Trp$^-$ synthetic medium to ensure that every cell contained pGBT9-TNFR-1IC. The overnight culture was sequentially transformed with 500 μg of a HeLa S3 cDNA library inserted into the two-hybrid activation vector pGAD GH. Doubly transformed cells were plated on 50 Leu$^-$, Trp$^-$, His$^-$ plates. The cells were incubated for 5 days at 30 °C before positive colonies were picked, restreaked onto triple minus plates, and assayed for the *lacZ* phenotype. Library clones from colonies that were His$^+$ LacZ$^+$ were isolated and retransformed alone, with pGBT9, with pGBT9-TNFR-1IC, and with pLAM5′. The library clones that activate the *lacZ* reporter gene only in the presence of pGBT9-TNFR-1IC were chosen for sequencing. Sequencing was conducted using the Sequenase sequencing kit (U. S. Biochemical Corp.).

Construction of Deletions of TNFR-1—Six primers were designed to amplify various regions within TNFR-1IC. All 5′ primers were linked to *Eco*RI and the 3′ primers were linked to *Bam*HI for easy and oriented cloning. All constructs were sequenced at the fusion sites to confirm in-frame fusion of TNFR-1IC and TNFR-1IC deletion mutants with pGBT9 and pGAD424.

Color Development Assays—Yeast harboring both GAL4 binding and activation domain fusion proteins were assayed for β-galactosidase activity using filter and liquid assay methods. For filter assays, yeast transformants were transferred to nitrocellulose filters, permeabilized in liquid nitrogen, and placed on Whatman No. 1 filter paper that had been soaked in Z-buffer (60 mM Na_2HPO4, 40 mM NaH_2PO4, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol) containing 1.0 mg/ml 5 bromo-4-chloro-3-indolyl β-D-galactoside at 30 °C. Positive colonies appeared in 5 min to 10 h. For the liquid assay, cultures were grown overnight in the appropriate synthetic media and then diluted 5-fold in rich media (YPD) and grown until mid-log phase (A_{600} of 0.4–0.8). Cells were then snap-frozen in liquid nitrogen, thawed at 37 °C, and then further disrupted by vortexing with glass beads. The procedure of Miller (27) was then used to quantitate β-galactosidase activity; however, chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim) was used for color development and cell pellets were resuspended in 900 μl of buffer H (100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 1% bovine serum albumin at pH 7.0). 100 μl of 50 mM chlorophenol red-β-D-galactopyranoside was added following cellular disruption and the amount of liberated chlorophenol red-β-D-galactopyranoside was determined by A_{574}. Numbers represent β-galactosidase activity in Miller units (27) and are expressed as the mean of triplicate determinations ± S.D.

TABLE I

β-Galactosidase activity and filter color of fusion constructs

Yeast strains SFY526 were transformed with various combinations of plasmids. β-galactosidase activity was assayed in SFY526 as described under "Materials and Methods."

Protein fused to GAL4 domain		β-Galactosidase activity	Filter color
DNA binding	Activating		
pVA3[a]	pTD1[a]	617.0 ± 41.7	Blue
pGBT-TNFR-1IC	None	0.1 ± 0.1	White
None	pGAD-TNFR-1IC	0.2 ± 0.0	White
pGBT-TNFR-1IC	pGAD-TNFR-1IC	23.7 ± 4.0	Blue
pGBT9	None	0.4 ± 0.1	White
None	pGAD424	0.6 ± 0.3	White
pGBT9	pGAD424	0.5 ± 0.1	White

[a] The plasmids pVA3 and PTD1 contain murine p53 and SV40 large T-antigen, respectively, interact strongly, and serve as a positive control.

RESULTS

TNFR-1IC was fused with the DNA binding domain and the transactivation domain of GAL4 to determine whether it is capable of self-interaction. Co-transformation of these plasmids into SFY526 activated the *lacZ* indicator gene and produced β-galactosidase activity (Table I). Yeast transformed with the DNA binding domain of GAL4 (pGBT9), the activation domain of GAL4 (pGAD424), the binding domain of GAL4 fused to TNFR-1IC (pGBT-TNFR-1IC), and the activation domain of GAL4 fused to the TNFR-1IC (pGAD-TNFR-1IC) by themselves were incapable of producing β-galactosidase activity. These plasmids were also transformed into HF7c and tested for His$^+$ prototrophy. Only pGBT-TNFR-1IC/pGAD-TNFR-1IC activated the *lacZ* reporter gene in SFY526 (Table I) and also activated HIS3 in HF7c (data not shown). Thus, only a hybrid in which interaction of the GAL4 binding and activation domains is mediated through aggregation of TNFR-1IC or a positive control in which p53 was fused to the GAL4 binding domain and the SV40 large T cell antigen was fused to the transactivation domain activated the *lacZ* reporter gene in SFY526 and HIS3 in HF7c.

To establish that aggregation of TNFR-1IC is specific, the ability of other proteins to interact with TNFR-1IC or one another was tested. The GAL4 binding domain and the GAL4 transactivation domain were incapable of interaction with TNFR-1IC (Table II). The ability of the intracellular domains of two other members of the TNF receptor superfamily (15), TNFR-2 and the FAS antigen, to interact with TNFR-1IC was evaluated. TNFR-2IC was incapable of self-association or interaction with TNFR-1IC, and experiments with the FAS antigen also were negative. Finally, two proteins unrelated to TNFR-1, lamin and p53, were unable to bind TNFR-1IC. These results support the view that aggregation of TNFR-1IC is specific. Further support for this conclusion is derived from the demonstration that experiments with two different yeast strains, SFY526 (Tables I and II) and HF7c (data not shown), with different GAL4 binding sites in the *lacZ* gene promoter region yielded comparable results.

Results from experiments in which a HeLa S3 cDNA library was screened for proteins that interact with TNFR-1IC, con-

TABLE II

Specificity of interactions in the two-hybrid assay

Yeast strains were transformed with various combinations of plasmids and then assayed for activation of β-galactosidase activity as described under "Materials and Methods."

Protein fused to GAL4 domain		β-Galactosidase activity in SFY526	Filter color
DNA binding	Activating		
pGBT-TNFR-1IC	pGAD-TNFR-1IC	23.7 ± 4.0	Blue
pGBT9	pGAD-TNFR-1IC	0.5 ± 0.1	White
pGBT-TNFR-1IC	pGAD424	0.6 ± 0.1	White
pGBT-TNFR-2IC	pGAD-TNFR-2IC	0.8 ± 0.4	White
pGBT-TNFR-1IC	pGAD-TNFR-2IC	0.5 ± 0.0	White
pGBT-TNFR-2IC	pGAD-TNFR-1IC	0.5 ± 0.2	White
pGBT-TNFR-1IC	pGAD-FAS-IC	0.5 ± 0.0	White
pGBT-FAS-IC	pGAD-TNFR-1IC	0.7 ± 0.3	White
pGBT-lamin	pGAD-TNFR-1IC	0.5 ± 0.1	White
pGBT-p53	pGAD-TNFR-1IC	0.7 ± 0.2	White

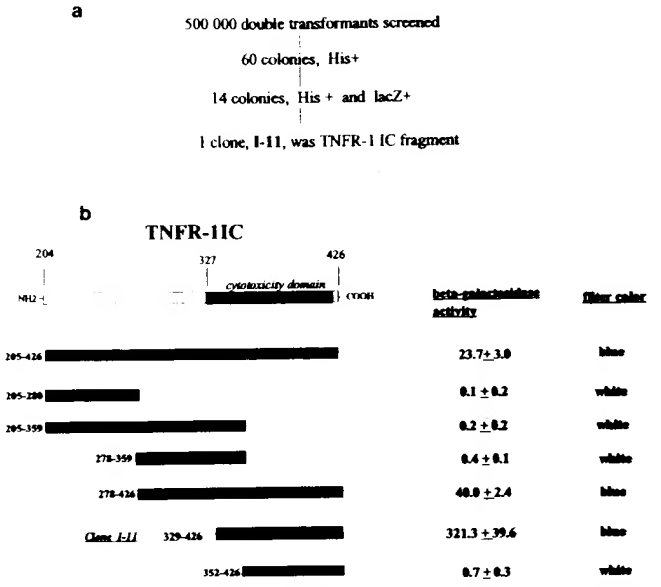

FIG. 1. *Panel a*, screening of a HeLa cell cDNA library. pGBT9-TNFR-1IC was used to screen a HeLa S3 cDNA library, which was cloned into pGAD GH as described under "Materials and Methods." Among the interacting proteins was one with amino acid sequences present in TNFR-1IC. *Panel b*, interaction of TNFR-1IC with TNFR-1IC deletion mutants. Deletion mutants in TNFR-1IC were prepared and inserted into the GAL4 activation domain plasmid pGAD424. pGBT9-TNFR-1IC and each activation domain fusion were co-transformed into SFY526 yeast, which was assayed for β-galactosidase activity. *Horizontal bars* represent the amino acid sequence of the complete intracellular domain of TNFR-1, the deletion mutant receptors, and the amino acid sequence identified from the library screen (clone I-11). The ability of each to activate β-galactosidase and filter color is indicated to the *right* of the figure.

firm self-association, and provide insight into the amino acid sequences in the receptor that mediate such interaction. Plasmids encoding TNFR-1IC fused with the GAL4 DNA binding domain (pGBT-TNFR-1IC) and proteins encoded by a HeLa cell cDNA library fused to the GAL4 trans-activation domain (pGAD GH-HeLa) were co-transformed into HF7c. 500,000 double transformant colonies were screened and selected for histidine prototrophy (Fig. 1*a*). 60 colonies were isolated as His$^+$ transformants. Very few transformants survived the HIS selection because most of the proteins in the activation domain fusions did not bind to the intracellular domain of TNFR-1. The His$^+$ transformants were assayed for *lacZ* expression to eliminate false positives. This procedure is effective as the *his3* and *lacZ* reporter genes in HF7c are under control of dissimilar promoters. The library DNAs from 14 double positive colonies were isolated and cotransformed with various control plasmids to demonstrate specificity of interaction with TNFR-1IC. All double positive clones showed specific interactions as evidenced by control experiments, which yielded results similar to those in Table II (data not shown). The cloned DNAs were sequenced, and one (I-11) encoded a protein domain in the C terminus of TNFR-1IC, which spans amino acids 329–426 (Fig. 1*b*). This observation shows that a small part of TNFR-1IC contains all of the recognition elements necessary for association. Furthermore, the protein encoded by this clone interacted more strongly with TNFR-1IC than did the full-length TNFR-1IC as evidenced by greater activation of β-galactosidase activity, 321 Miller units compared to 23.7. To better define the amino acid sequences important for aggregation, we constructed mutant receptors containing various C-terminal truncations (Fig. 1). These were subcloned into pGAD424 and cotransformed into SFY526 together with pGBT9-TNFR-1IC, which were assayed for interaction with TNFR-1IC based on activation of β-galactosidase. The inability of amino acids 205–280, 205–359, and 278–359 to interact with pGBT9-TNFR-1IC shows that sequence information in the first two-thirds of the N-terminal part of the intracellular domain is not necessary for aggregation. Amino acids 329–426 are the minimal domain thus far found to be necessary for aggregation. Further truncation to produce a peptide encompassing amino acids 352–426 resulted in loss of interaction with TNFR-1IC.

DISCUSSION

Tumor necrosis factor was first identified and characterized on the basis of its ability to induce the hemorrhagic necrosis and regression of tumors in experimental animals and by the cytotoxic response that it can elicit in transformed cells (1–3). Subsequent studies reinforced interest in TNF with the demonstration that it has profound effects on the growth, differentiation, and metabolism of non-transformed cells, is an important component of the host response to infection, and promotes immunity and metabolic alterations associated with various disease states (1–3). The identification and characterization of TNFR-1 and TNFR-2 and the cloning of cDNAs encoding each receptor have provided essential tools through which the proximal steps in TNF action may be investigated (8–14).

Numerous studies, most notably those employing receptor-specific antisera, have shown that the functions of TNF are segregated among the two receptors (10, 17–21). Activation of TNFR-1 elicits cytotoxicity in transformed cells (23, 24). An 80-amino acid domain near the C terminus of TNFR-1IC plays an obligatory role in signaling this response (22). The FAS antigen, another member of the TNF/NGF receptor superfamily that signals programmed cell death, contains a region with homology to the death domain in TNFR-1 (28). The importance of apoptosis to the oncolytic activity of cytokines and in developmental processes and the identification of a conserved domain essential to this function makes TNFR-1 an important model system.

Ligand-induced clustering of growth factor receptors is a mechanism through which receptor intracellular domains may interact and transactivate one another by autophosphorylation (29). A number of lines of evidence suggest that while TNF receptors do not possess intrinsic enzymatic activity, receptor aggregation may be an important component of signal transduction. First, TNF-α and TNF-β exist primarily as trimers in solution and in the crystal, which suggests three receptor binding sites (30–32). This is confirmed by the crystal structure of the complex between TNF-β and the extracellular domain of TNFR-1, in which three receptors are symmetrically bound to one TNF-β trimer (33). This structure, which is believed to be relevant to TNF-α as well as TNF-β, is predicted to bring the transmembrane domains into proximity and thereby allow the

cytoplasmic domains of the receptors to interact and generate the signal necessary for transmission of the TNF message to downstream structures. Second, the ability of antisera to the extracellular domain of TNFR-1 to elicit responses is consistent with aggregation (10, 17, 18). Third, the observation that monovalent Fab fragments against TNFR-1 do not induce cellular responses unless cross-linked with a second antibody suggests that aggregation plays a functional role in TNF action (10). Consistent with this premise is the dominant negative effect of TNFR-1 truncated in the cytoplasmic domain (23, 24). These mutated receptors associate with endogenous, full-length receptors and presumptively form non-functional complexes in which intracellular domains are incapable of interaction or signal transmission.

The two-hybrid system is a method that detects proteins capable of interacting with a known protein by transcriptional activation of a reporter gene (25, 26). This method has recently found wide application, for example, demonstrating interactions between Bcl-2 and R-Ras p23 (34), Sos 1 and GRB2 (35), Ras and Raf (36), and self-association of the retinoblastoma protein (37) and also the human immunodeficiency virus type 1 integrase (38). In the present study, the two-hybrid system was used to demonstrate that TNFR-1IC is capable of self-association. This interaction is specific as evidenced by the inability of unrelated proteins (lamin, p53, TNFR-2IC) and even proteins with sequence homology to TNFR-1IC (the intracellular domain of the FAS antigen) to activate the *lacZ* reporter gene under control of GAL4 transcriptional activation.

By using the two-hybrid system to screen a HeLa cell cDNA library for proteins capable of interaction with TNFR-1IC in conjunction with studies employing truncated receptors, we obtained further evidence for aggregation of TNFR-1 and mapped the amino acid sequences essential for self-association. The peptide containing amino acids 329–426 is the smallest necessary and sufficient for activation of β-galactosidase activity and therefore interaction with TNFR-1IC. Even a modestly smaller peptide (amino acids 353–426) was incapable of such binding. Peptides composed of amino acids 278–426 or 204–426 interacted less strongly with TNFR-1IC than did the minimal aggregation domain of amino acids 329–426. This may suggest that the conformation of the these longer peptides masks the aggregation domain, thereby diminishing receptor-receptor interactions. We speculate that TNF binding, which brings receptors into close apposition by virtue of its trimeric structure, also induces conformational changes permissive of interactions among TNFR-1IC aggregation domains, thereby promoting signal transmission.

Previous studies (22) have identified a domain at the C terminus of TNFR-1IC (amino acids 327–413) that plays an obligatory role in signaling cytotoxicity. Truncation to produce a peptide containing amino acids 341–426 abrogated the ability of the mutant receptor to induce a cytotoxic response in L-929 cells. Most striking is the similarity of the TNF receptor cytotoxicity domain with the domain (amino acids 329–426) now shown to mediate aggregation. The demonstration that a common domain contains elements essential to both aggregation and cytotoxicity suggests a functional relationship between these processes.

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STIC-ILL

From: Romeo, David
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Subject: 08/981,559 Boldin M P; Mett I L; Wallach D

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AU   Boldin M P; Mett I L; Wallach D
TI   A protein related to a proteasomal subunit binds to the
       ***intracellular***     ***domain***   of the p55   ***TNF***
     receptor upstream to its 'death domain'.
SO   FEBS LETTERS, (1995 Jun 19) 367 (1) 39-44.
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A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its 'death domain'

Mark P. Boldin, Igor L. Mett, David Wallach*

Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 19 April 1995; revised version received 8 May 1995

Abstract A novel protein that binds specifically to the intracellular domain of the p55 tumor necrosis factor (TNF) receptor was cloned by two hybrid screening of a HeLa cell cDNA library. Data bank searches revealed high sequence similarity of the protein (55.11) to yeast, nematode and plant proteins, whose functions are yet unknown. Significant similarity was also found between 55.11 and *SUG1*, the yeast equivalent of the p112 subunit of the 26S proteasome. Deletion analysis showed that the protein binds to the p55 receptor upstream to the region involved in induction of cell death.

Key words: Death domain; Receptor; Proteasome; Signaling; Tumor necrosis factor; Two hybrid system

1. Introduction

Triggering of the two TNF receptors, the p55 receptor (p55-R, CD120a) and the p75 receptor (p75-R, CD120b), initiates a wide range of effects, including modulation of differentiation patterns, activation of various immune mechanisms, induction of cell death, and stimulation of cell growth [1–3]. As with other receptors, these varied activities and their coordinated induction are likely to be effected by heterogeneity of functional motifs in the TNF receptors and of effector proteins with which the receptors interact. Currently, the majority of information available on the mechanisms of action of the TNF receptors concerns the C-terminal regions in the receptors' intracellular domains. In the p75-R, a region of about 78 amino acids in the C-terminus of the receptor is involved in the enhancement of T cell growth and NF-κB activation by TNF. Two proteins that bind to this C-terminal region (TRAF1 and TRAF2) probably take part in the involved signaling [4]. Studies on the mechanisms of action of p55-R have focused on a conserved sequence motif of about 80 amino acids at its C-terminus, which signals for cell death, and therefore, has been called the 'death domain' [5,6]. This motif, which is also present in some other proteins [7,8], can self-associate and bind to analogous sequences. The death domain of p55-R binds to itself and to a death domain motif present in Fas/APO1 (CD95), a structurally-related receptor that also signals for cell death. Also the death domain of Fas/APO1 self associates and binds to an analogous sequence in a recently cloned cytoplasmic protein, MORT1, which appears to participate in induction of cell death by Fas/APO1 [8,9]. Besides inducing cell death, the death domain of p55-R contributes to the induction of noncytocidal TNF effects, including anti-viral state and activation of the acid sphingomyelinase [6,10]. However, signaling for some TNF effects involves regions of p55-R other than its death domain. For example, the TNF induced activation of the neutral sphingomyelinase involves a region located upstream to the death domain, and the induction of nitric oxide synthase involves both the death domain and a region located upstream to it [6,10]. Besides being involved in signaling, the membrane proximal part of p55-IC participates in the regulation of uptake of the receptor [11]. We report here the cloning of a protein that binds to the intracellular domain of the p55-R (p55-IC) at a region upstream to its death domain.

2. Materials and methods

2.1. Cloning of the cDNA of 55.11

A partial cDNA of 55.11 (nts 925–2863, which encode for amino acids 309–900, see Fig. 4) was cloned by a two hybrid screen [12] of a Gal4 activation domain tagged HeLa cell cDNA library (Clontech, Palo Alto, CA, USA), as previously described [9]. The rest of the 55.11 cDNA (nts 1–924, which encode for amino acids 1–308) was cloned by PCR from a human fetal liver cDNA library. The nucleotide sequence of 55.11 was determined in both directions by the dideoxy chain termination method.

2.2. Two hybrid β-galactosidase expression tests

β-galactosidase expression tests were performed as previously described [8], except that in part of the tests, the pVP16 vector, which contains the activation domain of VP16, was used instead of pGAD-GH, the Gal4 activation domain vector. Numbering of residues in the proteins encoded by the cDNA inserts are as in the Swiss Prot data bank. Deletion mutants were produced by PCR, and point mutants by oligonucleotide directed mutagenesis [13].

2.3. Northern analysis

Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH), denatured in formaldehyde/formamide buffer, electrophoresed through an aparose/formaldehyde gel, and blotted to a GeneScreen Plus membrane (Dupont, Wilmington, DE, USA) in 10× SSPE buffer, using standard techniques. The blots were hybridized with the cDNA of 55.11 (nts 925–2863), radiolabeled with the random-prime kit (Boehringer Mannheim Biochemical, Mannheim, Germany), and washed stringently. Autoradiography was performed for 1 week.

2.4. Expression of 55.11 cDNA in HeLa cells and binding of the 55.11 protein to glutathione S-transferase fusion proteins of p55-IC

Glutathione S-transferase (GST) fusions with p55-IC (GST-p55IC) and with p55-IC truncated below amino acid 345 (GST-p55IC 345) were produced and absorbed to glutathione agarose beads as previously

*Corresponding author. Fax: (972) (8) 344165.
E-mail: IVWALLACH@WEIZMANN.WEIZMANN.AC.IL

The nucleotide sequence of 55.11 will appear in the GenBank/EMBL Data Bank under Accession Number X86446.

Abbreviations: GST, glutathione S-transferase; Fas-IC, intracellular domain of Fas/APO1; FLAG-55.11, the region extending between residues 309 and 900 in the 55.11 protein and which was N-linked to the FLAG octapeptide; IC, intracellular domain; p55-R, the p55 receptor for TNF; p75-R, the p75 receptor for TNF; p55-IC, intracellular domain of the p55-R; TNF, tumor necrosis factor.

SSDI 0014-5793(95)00534-X

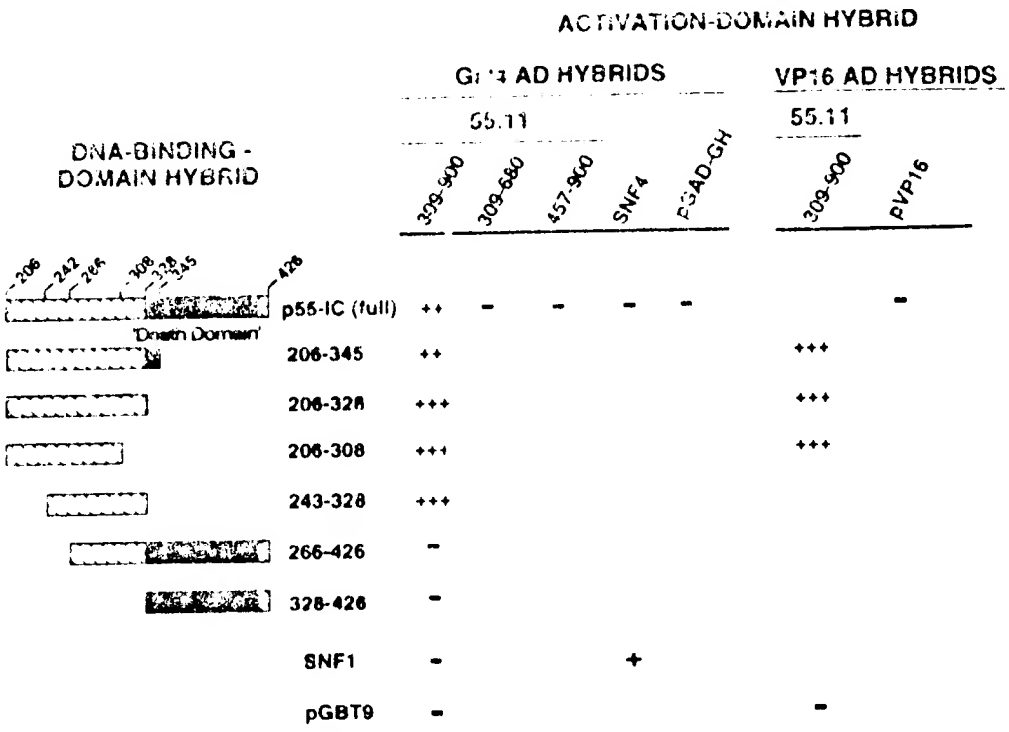


Fig. 1. Binding of the 55.11 protein to p55-IC within transformed yeast. Binding of human p55-IC (residues 206–426) and various deletion mutants to activation domain hybrids containing 55.11 was examined in transfected SFY526 yeast. Gal4 DNA binding domain constructs (pGBT9) and either Gal4 (pGAD GH) or VP16 (pVP16) activation domain constructs were used. The binding was assessed by a two-hybrid *β*-galactosidase expression filter assay. SNF1 and SNF4 served as positive controls and the empty Gal4 and VP16 vectors as negative controls. +++ and ++ indicate the development of strong color within 20 and 60 min of initiation of the assay, respectively. – indicates no development of color within 24 h. Blanks indicate not tested. The 55.11 protein did not bind to lamin, cyclin D, and the intracellular domains of human Fas/APO1 (residues 175–319), CD40 (residues 216–277), and p75 R (residues 287–461) (data not shown).

described [9,14,15]. The cDNAs of 55.11 (nts 1–2863), of FLAG-55.11, and of luciferase were expressed in HeLa cells. FLAG-55.11 is the region extending between residues 309 and 900 in the 55.11 protein (the partial cDNA of 55.11 (nts 925–2863) originally cloned by the two hybrid screen). N-linked to the FLAG octapeptide (Eastman Kodak, New Haven, CT). Expression of the fusion proteins was accomplished using a tetracycline-controlled expression vector (pTET-D) in a HeLa cell clone that expresses a tetracycline controlled transactivator [9,16]. Metabolic labeling of the expressed proteins with [^{35}S]-Met and [^{35}S]-Cys (Dupont, Wilmington, DE, and Amersham, Buckinghamshire, UK), lysis of the HeLa cells, immunoprecipitation, and binding of the labeled proteins to the GST-fusion proteins were performed as described before [8], except that 0.5% rather than 0.1% Nonidet P-40 was present in the cell lysis buffer. The immunoprecipitations of 55.11 and FLAG-55.11 were achieved using a rabbit anti-serum (diluted 1:500) raised against a GST-fusion protein containing the region of 55.11 that extends between amino acids 309 and 900, and a mouse monoclonal antibody against the FLAG octapeptide (M2, Eastman Kodak, 5 *μg*/ml) cell lysates.

3. Results and discussion

Since the death domain of p55-IC tends to self-associate [9], two-hybrid screens [14] for proteins that bind to p55-IC yield primarily the cDNA of p55-IC itself. However, when screening a HeLa cell library with p55-IC we isolated a cDNA clone (55.11) whose binding site to p55-IC appeared distinct from the death domain. The protein bound to a truncated p55-IC from which the death domain had been deleted (construct 206–328 in Fig. 1), more effectively than to nontruncated p55-IC. It also bound to an even further C-terminally truncated construct (construct 206–308) and to a construct from which both the death domain and a membrane proximal part were deleted (construct 243–328). However, the 55.11 protein did not bind to a construct that was N-terminally truncated down to amino

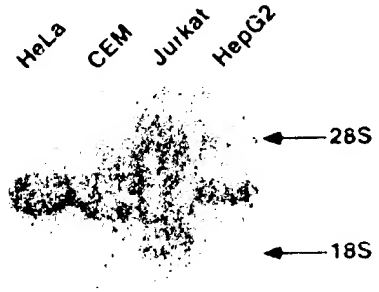


Fig. 2. Northern analysis of the RNA from several cells lines using the 55.11 cDNA as a probe. The cell lines examined were HeLa, CEM, Jurkat, and HepG2 cells derived from human epithelioid carcinoma, an acute lymphoblastic T cell leukemia, an acute T cell leukemia, and a hepatocellular carcinoma, respectively. The 55.11 cDNA (nts 925–2863) was used as a probe. Samples consisted of 40 *μg* of RNA/lane.

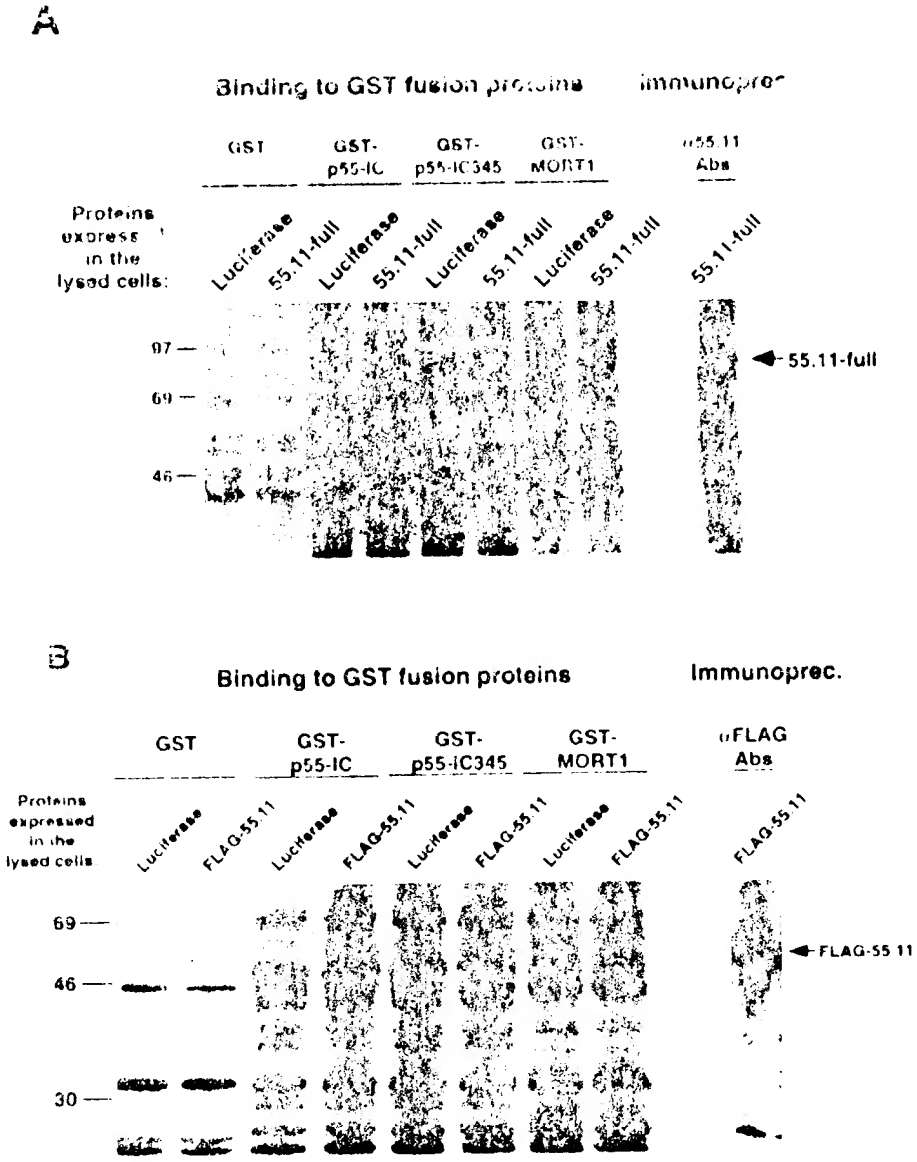
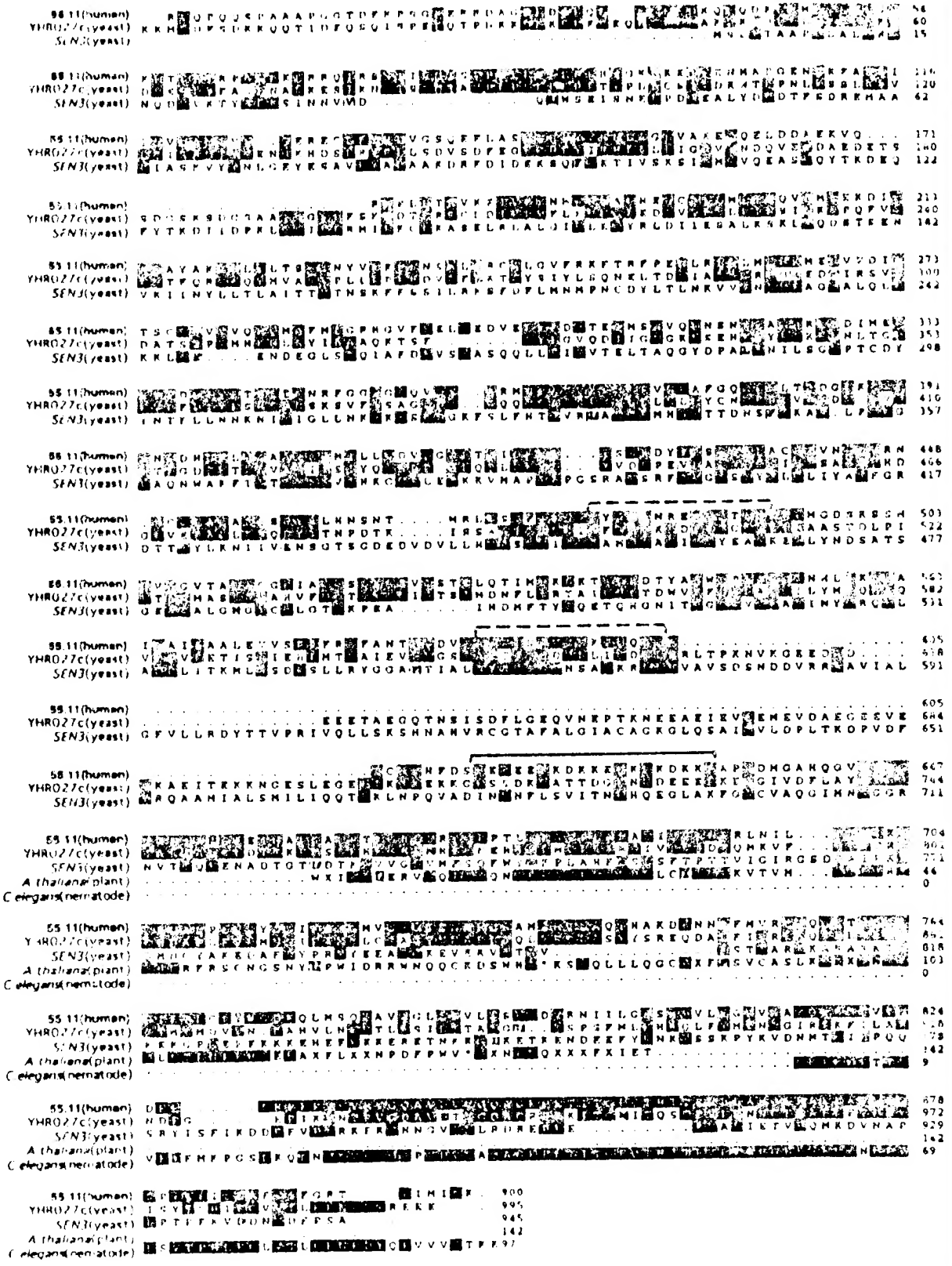


Fig. 3. In vitro binding of the protein encoded for by 55.11 to GST fusion proteins containing portions of p55-IC. Binding of the full length 55.11 protein (55.11-full) (A) and of FLAG-55.11 (amino acids 309–900 of 55.11, encoded by the initially cloned partial cDNA, fused at the N terminus with the FLAG octapeptide) (B). The cDNAs for the full-length 55.11, FLAG-55.11 and luciferase (control) were expressed in transfected HeLa cells and metabolically labeled with [^{35}S]Met and [^{35}S]Cys. The following proteins were fused with GST: full-length p55-IC (GST-p55-IC), p55-IC C-terminally truncated up to amino acid 345 (GST-p55-IC345), and the MORT1 protein (GST-MORT1). GST-MORT1 and GST alone served as controls. Lysates of the transfected cells were immunoprecipitated with antibodies against the 55.11 protein or the FLAG octapeptide. The proteins were analyzed by SDS polyacrylamide gel electrophoresis (10% acrylamide) followed by autoradiography.

acid 266 (Fig. 1). These findings indicate that the binding site for 55.11 is located in the region that extends between residues 243 and 308 of p55-IC and that the N terminus of this binding site is between residues 243 and 266.

Transfer of the cDNA for 55.11 from the originally cloned 'prey' construct, which contained the Gal4 activation domain, to a prey construct containing the VP16 activation domain did not decrease the binding efficiency of the 55.11 protein to p55-IC (Fig. 1). Thus, the structure(s) involved in this binding appear to reside within the 55.11 molecule and not to involve the site of fusion of 55.11 with the activation domain. However, binding of 55.11 to p55-IC was abolished by even limited truncations of the 55.11 protein at either its C (construct 309–680) or N terminus (construct 457–900). Residue 309 is the first residue in the protein encoded by the partial cDNA clone originally isolated in the two hybrid screen). The observed binding


55.11(human)
YHR027c(yeast)
SEN3(yeast)
A.thaliana(plant)
C.elegans(nematode)

appeared to be specific since 55-H did not bind to other proteins, including three receptors of the TNF/NGF receptor family (p75-R, Fas/APO-1 and CD40, data not shown).

Northern analysis using the 55-H cDNA as a probe (Fig. 2) revealed, in several cell lines, a single hybridizing transcript of about 3 kb, which is larger than the cDNA (2 kb). Using oligonucleotide primers that correspond to the 55-H sequence, we cloned by PCR a 5′ extending sequence whose length was about 1 kb. The sum of the length of this 5′ extending sequence with that of the originally cloned cDNA approximates the length of the 55-H transcript. The 3 kb cDNA that encompassed both these portions was effectively expressed in transfected HeLa cells yielding a protein of about 84 kDa, which suggests that the 3 kb cDNA contains a translational start site.

To ascertain that 55-H can indeed bind to p55-IC and to exclude involvement of yeast proteins in this binding, the in vitro interaction of GST–p55-IC fusion proteins, produced by bacteria, with the protein encoded by the 3 kb 55-H cDNA (55-H full), produced by transfected HeLa cells, was examined. The protein encoded by 55-H bound to fusion proteins that contained the full p55-IC (GST–p55IC) or a truncated p55-IC that lacked most of the death domain (GST–p55IC 345) (Fig. 3A). The protein did not bind to GST alone or to GST fused to the MORF1 protein (control). Similarly, the HeLa cell expressed protein encoded by the initially cloned partial cDNA of 55-H in fusion with the FLAG octapeptide (FLAG–55-H) bound in vitro to GST–p55IC and GST–p55IC 345, but not to GST or GST–MORF1 (Fig. 3B).

Data bank searches revealed that parts of the sequence of the 55-H cDNA (Accession Numbers T03689, Z19559, and T09128) and its mouse homologue (Accession Numbers X80422 and Z31447) have already been determined during arbitrary sequencing of cDNA libraries. A cDNA sequence (Accession Number U18247) that encodes for a human protein of 896 amino acids present in cultures of human hepatoma HC-10 cells is almost identical to that of 55-H. This hepatoma protein lacks an N-terminal portion (amino acids 1–297) corresponding to that of 55-H and also differs from 55-H at the regions that correspond to residues 297–372 and residues 648–668 in 55-H. The searches of the data bank also revealed that proteins with very high sequence homology to 55-H exist in *Saccharomyces cerevisiae* (yeasts), *Arabidopsis thaliana* (plants) and *Caenorhabditis elegans* (worms). In yeast, there are two known proteins whose DNA sequences resemble that of 55-H (the open reading frame YHR027c and *SIN3*, Fig. 4). The sizes of both are close to that of 55-H. YHR027c is known only by the sequencing of a genomic clone while *SIN3* has been cloned as a cDNA. The sites within 55-H that are similar to those in *SIN3* correlate to the sites of its similarity to YHR027c, although much more similarity is evident between 55-H and YHR027c than between 55-H and *SIN3*. The DNA sequence information available for the *Arabidopsis thaliana* and *Caenorhabditis elegans* proteins, although only partial, clearly shows that these proteins are as similar to 55-H as the YHR027c protein of yeast. The only one of these four proteins whose nature has been elucidated so far is the yeast *SIN3*, whose homology to 55-H is limited. *SIN3* has been identified as the yeast equivalent of the p112 subunit of an activator of the 20S proteasome (the proteolytic core of the 26S proteasome [17,18]) (M.R. Culbertson and M. Hochstrasser, personal communication).

Conserved amino acid sequence motifs were not discerned within the protein encoded for by 55-H, except for a repetitive 'KEKE' sequence that extends between Lys^{433} and Glu^{457}. Such 'KEKE' sequences, which are present in many proteins, including proteasomal subunits and chaperonins, may promote association of protein complexes [19]. A sequence AYAGS(x)LL appears twice in the 55-H protein (at sites 479 and 590, see Fig. 4), no functional significance for this sequence has yet been described.

Findings in this study suggest several possible routes that could be explored to elucidate the function served by the 55-H protein and how its binding to the p55-R contributes to TNF activity. The finding that the 55-H protein is bound to a region in the p55-R distinct from the death domain indicates that it affects noncytocidal TNF activities). There are indications that the region to which the 55-H protein binds contains sequence motifs involved in TNF-induced expression of nitric oxide synthase [6] and activation of neutral sphingomyelinase [10]. However, these motifs have not yet been fully defined. The region is particularly rich in proline, serine, and threonine residues, yet it does not contain the RPM1 and RPM2 proline-rich motifs, present in several other cytokine receptors [20]. Proline residues follow two serines and two threonines in the N-terminal part of the binding region of the 55-H protein, making them potential sites for phosphorylation by MAP kinase, CDC2, and other proline-dependent kinases [21], which may affect receptor binding by the 55-H protein. More accurate definition of the sequence elements within the p55-R that are involved in 55-H binding, and those involved in the effects of TNF on nitric oxide synthase and neutral sphingomyelinase, will help clarify the role of the 55-H protein in signaling.

The marked sequence similarity of the 55-H protein with *SIN3*, the yeast equivalent of the p112 protein, provides additional clues to the function of the 55-H protein. If, as does the p112 protein, the 55-H protein occurs in the regulatory complex of the 26S proteasome, than its binding to p55-R may modulate the proteolytic function of the proteasome, or conversely, contribute to the degradation of the p55-R itself within the proteasome. Of note, interferon-γ, whose activities are closely related to those of TNF, has pronounced effects on the composition and function of the proteasomes [24,25]. Furthermore, although there is no information regarding how the TNF receptors are degraded, the rapidity with which TNF binding decreases after inhibition of protein synthesis, suggests that these molecules have a very short half-life, consistent with the

Fig. 4. Comparison of the deduced amino acid sequence of human 55-H to that of related proteins present in lower organisms. The sequences of amino acids predicted for the 55-H cDNA, an open reading frame (YHR027c) within a cosmid derived from the 8th chromosome of *Saccharomyces cerevisiae* (nts 21453–24354, Accession Number U10399), *SIN3*, the cDNA of a *Saccharomyces cerevisiae* protein (Accession Number U06321), a partial cDNA of a protein of the plant *Arabidopsis thaliana* (Accession Number T21700), and a partial cDNA of a protein of the nematode *Caenorhabditis elegans* (Accession Number D27899). The 'KEKE' sequence in 55-H is marked with a solid line and the sequence AYAGS(x)LL with broken lines. The sequences were aligned using the PILEUP and PRETTYBOX programs of the GCG package. Gaps introduced to maximize alignments are denoted by dashes.

possibility that their degradation occurs in the proteasomes (unpublished data). Additional studies of the significance of the sequence similarity between the 55 kD protein and the p112 protein, and of the particular function of the p112 unit within the proteasome, will help clarify the functional significance of the 55 kD protein interaction with the p55-R.

Although TNF itself has only been found in multicellular organisms, the occurrence of proteins closely related to 55 kD in yeast, as well as in plants and nematodes, indicates that this TNF-receptor associated protein serves an evolutionary conserved function. This is not surprising since a number of known molecules and mechanisms regulated by TNF seem to have appeared rather early in the evolutionary process. For example, several protein kinases known to signal TNF effects, as well as transcriptional factors affected by these kinases, also occur in yeast where they have important roles in signaling [21–23]. The technical advantage of the genetic set up in yeast for studying the function of an unknown protein, coupled with the advantage of the cellular composition of nematodes for studying the role of an unknown protein in a multicellular organism, will facilitate elucidation of the function of the 55 kD protein.

Acknowledgments: We wish to thank Dr. Jacques H. Camonis for his advice in applying the two hybrid system technique, Dr. M.R. Culbertson for providing unpublished information of the nature of *SEN3*, Ms. Tanya Gonchorov for her assistance in raising antibodies against the 55 kD protein, and Dr. Barbara Schick for her advice on the manuscript. This work was supported in part by grants from Inter-Lab Ltd. (Ness Ziona, Israel), Ares Trading S.A. (Geneva, Switzerland), and the Israeli Ministry of Arts and Sciences.

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TI   A cytotoxic CD40/p55   ***tumor***     ***necrosis***
       ***factor***   receptor hybrid detects CD40 ligand on herpesvirus
     saimiri-transformed T cells.
SO   EUROPEAN JOURNAL OF IMMUNOLOGY, (1995 Jan) 25 (1) 80-6.
     Journal code: EN5. ISSN: 0014-2980.
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Sigrun Hess ▼, **Roland Kurrle**, **Leander Lauffer**, **Gert Riethmüller** and **Hartmut Engelmann**

Institute for Immunology, University of Munich, Munich and Behringwerke AG, Preclinical Research, Marburg

A cytotoxic CD40/p55 tumor necrosis factor receptor hybrid detects CD40 ligand on herpesvirus saimiri-transformed T cells*

The B cell activation molecule CD40 and the p55 tumor necrosis factor receptor (p55TNFR) belong to the same family of structurally conserved proteins. We constructed a chimeric receptor consisting of the CD40 extracellular and transmembrane domains and the p55TNFR intracellular domain. This receptor hybrid retained the biological activity and the ligand specificity of the respective wild-type receptor domains. Thus it exerted a marked cytotoxic effect in three different transfected cell lines after activation not only with anti-CD40 antibody but also with CD40 ligand (CD40L) in soluble and membrane-bound forms. Using hybrid-transfected baby hamster kidney cells we demonstrated that herpesvirus saimiri-transformed human $CD4^+$ T lymphocytes constitutively express bioactive CD40 ligand on their surface. The hybrid receptor-based assay was highly specific for CD40 activating reagents and more sensitive than an assay measuring CD40-mediated B cell rescue from apoptosis. Hence CD40/p55TNFR transfectants may be useful for dissecting CD40L-mediated events in T-B cell interactions, and also to detect a defective CD40L molecule in putative hyper-IgM syndrome patients.

1 Introduction

Specific T-B lymphocyte interaction is critical for a coordinated humoral immune response. Key players in this interaction are two recently identified molecules, CD40 and its ligand. The CD40 ligand (CD40L), a 33-kDa type II transmembrane glycoprotein [1–3] is predominantly expressed on activated $CD4^+$ T lymphocytes [4–7] but can also be found on basophil and mast cell lines [8]. Naturally occurring mutations in the CD40L gene and gene targeting experiments demonstrate the central role of the CD40L-CD40 interaction in B cell immunity. Patients carrying CD40L mutations suffer from an immunodeficiency known as hyper-IgM syndrome. Because of a nonfunctional CD40L these patients fail to develop germinal centers in lymphatic tissues and isotype switching in their B lymphocytes is severely impaired [9–13].

In vitro studies demonstrate that CD40 activation via its ligand or mimetic Ab influences other important B cell functions besides isotype switching [14–17]. Thus CD40 co-stimulates short-term proliferation [16, 18–20] and long-term proliferation [21, 22] and enhances B cell survival. The anti-apoptotic activity of CD40 was shown in germinal center B cells [23] and in a subgroup of Burkitt lymphoma cell lines [24, 25]. The expression pattern of CD40, however, indicates that the biological role of this receptor is not restricted to B cell immunity. The 50-kDa molecule is also expressed on dendritic cells [26], thymic epithelium [27], monocytes [28], T lymphocytes [29, 30], basal epithelium [31] and on some carcinomas [31–34].

Both CD40 and its ligand belong to receptor and ligand gene families which include as prototypes the p55 TNF receptor (p55TNFR) and its ligands TNF or lymphotoxin-α (LT-α) [35, 36]. In contrast to the anti-apoptotic activity of the CD40 molecule, a major function of the p55TNFR is the induction of cell death. This is surprising in view of the structural homologies in the extracellular domains and in the signal transducing cytoplasmic regions of both receptors [34, 37–40] which might in fact indicate a close functional relationship.

Here we report that the similarities between CD40 and the p55TNFR are sufficient to allow the construction of a biologically active receptor hybrid. The chimeric receptor transfected into three different cell lines induces cell death upon activation with natural or recombinant CD40L and mimetic anti-CD40 Ab. These transfectants are by far more sensitive for detection of CD40 activating reagents than a conventional B cell assay. Using CD40/p55TNFR hybrid transfectants were demonstrate that herpesvirus saimiri (H. saimiri) transformed human $CD4^+$ T lymphocytes constitutively express functional CD40L on their surface.

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* This work was supported by grants from the Deutsche Forschungsgesellschaft (Gerhard Hess Programm and SFB217).
▼ Recipient of a postdoctoral fellowship from the Boehringer Ingelheim Fonds.

Correspondence: Hartmut Engelmann, Institute for Immunology, Goethestr. 31, D-80336 München, Germany (Fax: 89-51602236)

Abbreviations: **CD40L:** CD40 ligand **LT-α:** Lymphotoxin-α **ECD:** Extracellular domain **TMD:** Transmembrane domain **ICD:** Intracellular domain **BHK:** Baby hamster kidney cells **CHX:** Cycloheximide

Key words: CD40 / Tumor necrosis factor receptor / CD40 ligand / Herpesvirus saimiri

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2 Materials and methods

2.1 Cells

The murine L cell derivative A9 [41], the B cell lines IM-9 (ATCC CCL 159) and BL41 (kindly provided by Dr. G. Bornkamm) [42] and the histiocytic lymphoma cell line U-937 (ATCC CRL 1593) were grown in RPMI; the SV40-transformed human fibroblast cells SV80 [43] were cultured in Dulbecco's modified Eagle's medium, baby hamster kidney (BHK) cells (DSM ACC 61) in MEM (Eagle) with Earle's salts plus nonessential amino acids (Biochrom, Berlin, Germany), chinese hamster ovary (CHO dhfr) cells (ATCC CRL 9096) in α medium (Biochrom). For the H. saimiri-transformed cell lines CB15 [44] and Kesting [45], which were generously provided by Drs. Fickenscher and Fleckenstein, a mixture of 45 % CG-medium (Vitromex, Vilshofen, Germany), 45 % RPMI and 40 U/ml proleukin (Eurocetus GmbH, Frankfurt, Germany) was used [46]. All media were supplemented with 10 % heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-alanyl-L-glutamine. All supplements and culture media except CG- and α medium were purchased from Gibco BRL (Eggenstein, Germany).

2.2 CD40/p55TNFR hybrid receptor construction and transfection

Total RNA was isolated from IM-9 and U-937 cells [47] and transcribed into cDNA using superscript reverse transcriptase (Gibco BRL). The complete CD40 cDNA was then PCR-amplified from the IM-9 cDNA using the 5′ primer AATCTAGATGCCGCCTGGTCTCACCTCG and the 3′ primer AAAAGCTTGCCAACTGCCTGTTTGCCCACG, containing a 5′ XbaI or a 3′ HindIII restriction site, respectively. The PCR product was subcloned into pBluescript SK II(+)(Stratagene, La Jolla, CA) and designated BS-CD40. The cDNA coding for the TNFR intracellular domain (ICD) was amplified from U-937 cDNA using the 5′ primer AATGGCCAAGCTCTACTCCATTGTTTGT and the 3′ primer TTGCTAGCGCTCGAGCTCTAGAGCCTCATCTGAGAAGACTGG, containing additional XbaI and XhoI site at the 3′ end and was subcloned into pBluescript SK II(+) between EcoRV and XhoI (designated BS-TNFR-ICD). The sequence of all PCR products was confirmed by sequencing of both DNA strands.

To construct the CD40/TNFR hybrid cDNA, BS-CD40 was cut with BalI and XhoI and the cDNA coding for the CD40 ICD was replaced by the TNFR ICD fragment cut with BalI and XhoI. The cDNA encoding the CD40/TNFR hybrid receptor was then ligated into the mammalian expression vector pEF-BOS [48] within XbaI sites, designated BOS-CT.

For transfection by lipofection (Lipofectin, Gibco BRL), 50 % confluent A9, SV80 and BHK cells grown in 9.4-cm petri dishes (Greiner, Nürtingen, Germany) were co-transfected with 25 μg of ApaLI-digested BOS-CT and 2.5 μg of HindIII-digested pTCF plasmid [49] carrying a neomycin-resistance gene. Neomycin-resistant clones were selected in appropriate concentrations of G418 (Gibco BRL), 0.6 mg/ml for A9, 0.8 mg/ml for SV80 and 2 mg/ml for BHK cells. Expanded clones were immunostained using 5 μg/ml anti CD40 mAb (Laboserv) or anti CD4 mAb as an isotype-matched control and FITC-conjugated goat anti-mouse $F(ab')_2$ as secondary antibody. Hybrid receptor expression was then assessed by flow cytometry (FCM) (FACScan, Becton Dickinson).

2.3 Expression of CD40L in BHK cells

Human CD40L cDNA [2] was isolated by PCR from a Con A stimulated PBL cDNA library (Dr. Bernhard Knapp, Behringwerke) with the 5′-primer TGCCACCTTCTCGAGCAGAAGATACCATTTCAACTTTAAC and the 3′-primer GTCAGCTCCACCACCGGCCGCAAGGTGACACTGTTCAGAG. The resulting 665-bp fragment was ligated into the mammalian expression vector CDM8 [50] within XhoI and EagI restriction sites to result in CDM8-CD40L. BHK cells were cotransfected by Ca/PO_4 coprecipitation with CDM8-CD40L and the DHFR and neomycin selection vectors pSV4dhfr and pRMH140 according to Zettlmeisl et al. [51]. Colonies resistant to 200 nM methotrexate and 400 μg/ml G 418 were selected. A clone showing strong membrane fluorescence after staining with a CD40/Ig fusion protein (L. Lauffer, unpublished) and FITC-labeled goat anti-human Ig antiserum, designated BHK_{CD40L}, was used for further experiments.

2.4 Production of a IL-4R/CD40L fusion protein

The cDNA encoding the extracellular region of CD40L (CD40L ECD) was PCR-amplified from CDM8-CD40L with the 5′-primer CGAAGCTTGGATCCGAGAAGGTTGGACAAGATAGAAGATand the 3′-primer CGCTCTAGATGTTCAGAGTTTGAGTAAGCC. To generate the fusion protein we used a CD4/Ig fusion plasmid described earlier [52], replaced the CD4 sequence with the cDNA encoding the naturally occurring soluble form of the murine IL-4R [53] and exchanged the sequence encoding the Ig portion with the CD40L ECD PCR fragment. The resulting plasmid pmuIL-4R/CD40L encoded a fusion protein consisting of the soluble form of murine IL-4R including Leu^{205} [53] followed by primer-encoded aspartic acid and proline residues and the CD40L ECD from Arg^{48} [2] to the CD40L stop codon. Stably transfected BHK cells were generated as described above and expression levels of isolated clones were assessed using an IL-4R-specific ELISA with two murine mAb (R. Kurrle, unpublished). A clone expressing approximately 1–3 μg/ml of IL-4R/CD40L was chosen for production in roller bottles. Protein was purified from conditioned medium by immunoaffinity chromatography on an anti-murine IL-4R mAb column.

2.5 Cytokines

Recombinant human TNF and LT-α were a generous gift from Dr. G. Adolf (Bender and Co, GmbH, Wien, Austria).

2.6 Antibodies

Anti-CD40 rabbit antiserum was raised against a CD40 ECD fusion protein (CD40-ECD-His). In order to facili-

tate the purification of this protein, a DNA adapter 5′-CATCATCATCATTGATGAA-3′ plus 5′-AGCTTTCATCAATGATGATGATGATGGGCT-3′ encoding five histidines was ligated 3′ of the BanII restriction site into the cDNA coding for the CD40 ECD. The resulting cDNA was then inserted into a derivative of the DHFR-expression vector pMT2PC [54] kindly provided by Dr. P. Kufer (Institute for Immunology, Munich, Germany). DHFR-deficient CHO cells were transfected by electroporation (260 V, 960 μF), selected in nucleoside-free α medium plus supplements and the expression was amplified by adding up to 500 nM methotrexate (Sigma). Soluble CD40-ECD-His was purified from the cell supernatant using Ni-NTA Agarose (Diagen, Hilden) and reverse-phase HPLC. Two further antisera were raised against the soluble IL-4R/CD40L fusion protein or soluble CD14, respectively (Philip Bufler et al., manuscript in preparation). All antisera were obtained by a four-step immunization procedure with 30 μg/ml protein in PBS mixed with Freund's adjuvant at a ratio of 1:2. The anti-CD40 mAb BB20 was purchased from Laboserv (Giessen, Germany). The control anti-CD4 (MT413) and anti-CD14 (MEM 18) mAb were gifts from Dr. E. P. Rieber (Institute for Immunology, Munich, Germany) and Dr. V. Hořejší (Institute of Molecular Genetics, Prague, Czech Republic), respectively.

2.7 Cytotoxicity assay

Target cells were seeded in flat-bottom microtiter plates (Greiner) at a density of 4×10^4 cells/well, grown for 24 h and then challenged with serial dilutions of the stimulation reagents in the presence of 50 μg/ml CHX (Sigma). After 16–18 h, cell viability was assessed by neutral red uptake (Sigma) according to Finter [55]. The effector cells BHK_{CD40L} or BHK_{wt} were detached with 5 mM EDTA in PBS, fixed with 3% paraformaldehyde in PBS for 10 min and washed six times, before they were added to the target cells. This procedure prevented the attachment of the effector cells to the microtiter plates and the interference with the determination of the target cell viability. CB15 cells as effector cells were added natively as they are nonadherent T cells and could be removed from the targets by extensive washing.

2.8 B cell rescue assay

BL41 cells (1×10^5/well) were seeded in U-shaped microtiter plates (Greiner). Ionomycin (2 μg/ml; Sigma) and serial dilutions of the CD40 activating reagents were added simultaneously. BHK cells used as stimulators were fixed in the same manner as for the cytotoxicity assay. After 30 h incubation time the BL41 cells were stained with neutral red as described above.

3 Results

3.1 Hybrid receptor construction and transfection in A9, SV80, and BHK cells

In view of their different biological functions the structural conservation in CD40 and the p55TNFR is surprising. Such conserved structures may merely represent evolutionary relicts from a common ancestral molecule. Alternatively they may support a particular mode of receptor activation common to both receptors. In order to test the latter hypothesis, we constructed a chimeric receptor consisting of the ECD and TMD domains of CD40 and the intracellular domain of the p55TNFR (Fig. 1A). The hybrid receptor was expressed in the human SV80 and the mouse A9 fibroblast cell lines which are both highly sensitive for the cytotoxic effect of TNF. In addition we transfected the receptor chimera into BHK cells which are resistant to cytolysis by TNF but can be rendered sensitive by overexpression of the human p55TNFR [56].

Hybrid receptor expressing clones were obtained from all three cell lines and designated $A9_{CT}$, $SV80_{CT}$ and BHK_{CT} (Fig. 1B). Expression in the transfectants was approximately ten times above background. No changes in the responsiveness to TNF or LT-α were observed due to the transfection (Fig. 2). $A9_{CT}$ and $SV80_{CT}$ were sensitive to the cytotoxic effect of both cytokines whereas the BHK_{CT} cells remained as resistant as the corresponding wild-type cells (Fig. 2). Even cell-specific differences in the responsiveness to TNF and LT-α observed for SV80 wild-type cells could be shown in the hybrid-expressing clones and in neomycin-resistant control cells. These two SV80 clones responded

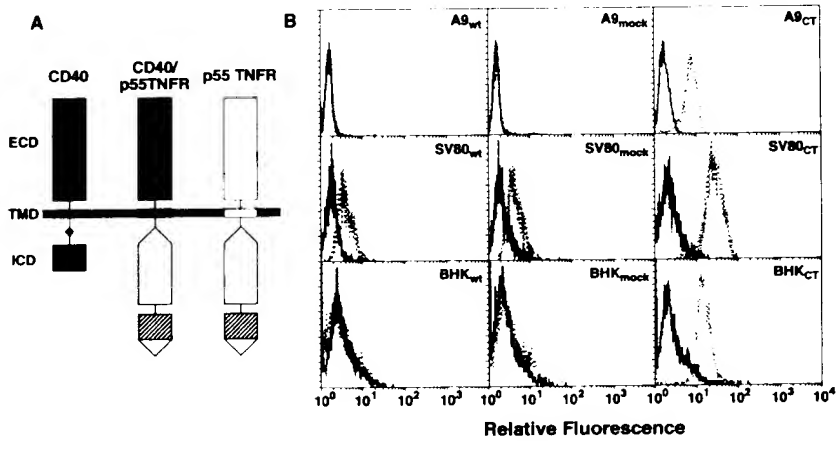

Figure 1. (A) Schematic presentation of CD40, the p55TNFR and the CD40/p55TNFR hybrid receptor. CD40 domains are shown in dark grey, those taken from the p55TNFR in white. Hatched parts indicate a homologous region of 53 aa present in the cytoplasmic domains of both wild-type receptors. (B) Expression of the hybrid receptor in mouse A9 fibroblasts, human SV80 fibroblasts and BHK cells. Wild-type cells, mock or hybrid receptor (CT) clones of the indicated cell types were immunostained using anti-CD40 mAb (BB20) (·····) or anti-CD4 mAb (MT413) (——) as an isotype matched control and FITC-conjugated goat anti-mouse $F(ab')_2$Ab. Stained cells were analyzed by FCM.

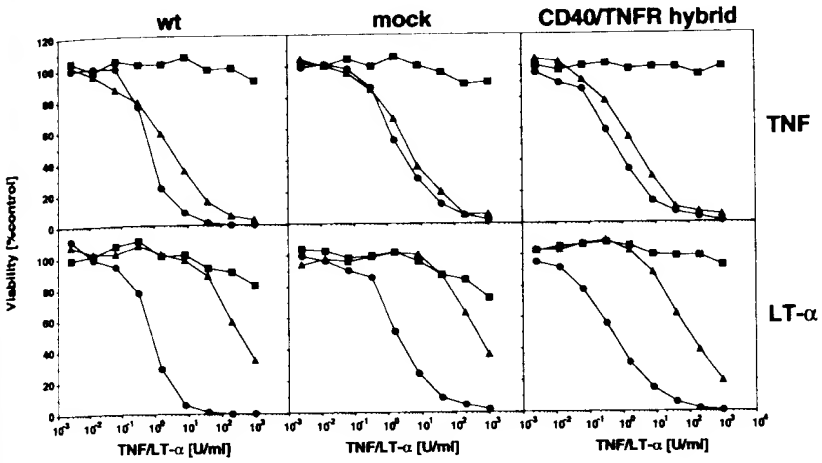

Figure 2. Sensitivity of hybrid receptor (CT)-expressing cells to TNF and LT-α. Wild-type cells, mock and CT clones of the human SV80 fibroblast (▲), the mouse A9 fibroblast (●) and the BHK (■) cell lines were treated with TNF or LT-α at the indicated concentrations in the presence of CHX (50 μg/ml). After 16 to 18 h incubation time viability was determined by the neutral red uptake method as described in Sect. 2.7 and is expressed as the percentage of control cells incubated with CHX alone.

better to TNF than to LT-α, reconfirming that transfection did not alter the responsiveness of the cells towards signals transduced by the p55TNFR.

3.2 The CD40/TNFR hybrid mediates cytotoxicity when activated by anti-CD40 Ab

In order to test the functional competence of the CD40/TNFR chimera we treated the transfectants with various anti-CD40 antibodies in the presence of CHX. As shown in Fig. 3A and B, all anti-CD40 Ab exerted cytotoxic activity on the hybrid transfectants (right panels in Fig. 3A and B, filled symbols) and were non-cytotoxic on the wild-type cells (not shown) and the mock clones (left panels in Fig. 3A and B). Killing was dose dependent with a half-maximal effect at serum dilutions of 1:30000 ($A9_{CT}$ and BHK_{CT}) and 1:150000 ($SV80_{CT}$) for the rabbit antiserum and at Ig concentrations of 1–3 ng/ml for the mAb BB20. Similar results were obtained with five other mAb directed against three different epitopes of CD40 (R. Schwabe et al., manuscript in preparation). Control antibodies (rabbit anti-CD14 Ab or an anti-CD14 mAb) were not cytotoxic on any of the cells (right panels in Fig. 3A and B, open symbols).

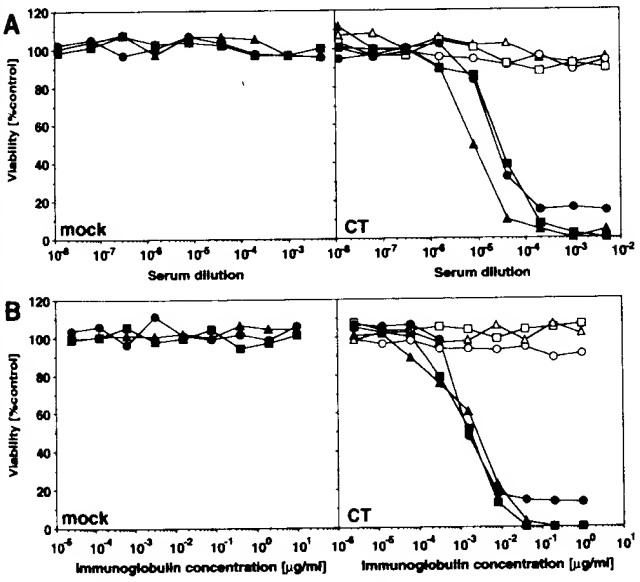

Figure 3. Anti-CD40 Ab are cytotoxic on hybrid receptor (CT)-expressing clones. (A) Mock and CT clones of the human SV80 fibroblast (▲, △), the mouse A9 fibroblast (, ○) and the BHK (■, □) cell lines were treated with rabbit anti-CD40 (▲, , ■) or rabbit anti-CD14 pAb (△, ○, □) as a control in the presence of CHX (50 μg/ml). Cell viability was determined 16 to 18 h later by neutral red staining. (B) Anti-CD40 (BB20) (▲, ●, ■) or anti-CD14 mAb (MEM18) (△, ○, □) were assayed under the same conditions as described in (A).

3.3 CD40L, the natural ligand of CD40, triggers the chimeric receptor

We next asked whether the hybrid receptor could be stimulated by its natural ligand, CD40L. First we tested the activity of the soluble IL-4R/CD40L fusion protein. Like anti-CD40 Ab, the soluble CD40L construct efficiently killed all three hybrid expressing clones (Fig. 4, right panel). The cytotoxicity was concentration dependent and reached half-maximal levels at concentrations of 20 ng/ml on $A9_{CT}$, 5 ng/ml on $SV80_{CT}$ and 100 ng/ml on BHK_{CT}, respectively. No cytotoxic activity was detectable on the corresponding wild type (not shown) or the neomycin-resistant control cells (Fig. 4, left panel). As the CD40L

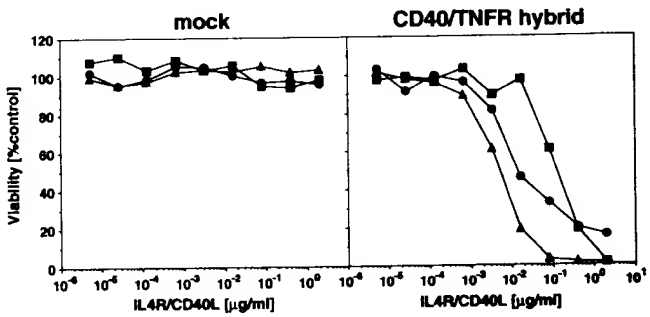

Figure 4. Soluble CD40L activates the CD40/TNFR hybrid receptor (CT). Mock and CT clones of the human SV80 fibroblast (▲), the mouse A9 fibroblast ( ) and the BHK (■) cell lines were treated with a soluble fusion protein consisting of the human CD40L and the murine IL-4R ECD in the presence of CHX (50 μg/ml). After an incubation time of 16 to 18 h the cell viability was determined by the neutral red uptake method.

predominantly exists in a membrane-bound form, it was interesting to examine whether this form could also activate the chimera. Therefore we tested BHK transfectants expressing CD40L on their cell surface (BHK_{CD40L}) (Fig. 5, left panel) for their cytotoxic activity on $A9_{CT}$ target cells. As shown in Fig. 5 (right panel), BHK_{CD40L} transfectants efficiently killed $A9_{CT}$ targets in a concentration-dependent manner. A killer to target ratio of 1:10 was sufficient to obtain 50 % killing. These results demonstrated that the cell surface form of CD40L also triggers the hybrid receptor. No cytotoxic effect was observed when $A9_{mock}$ cells were used as targets (Fig. 5, right panel, open symbols).

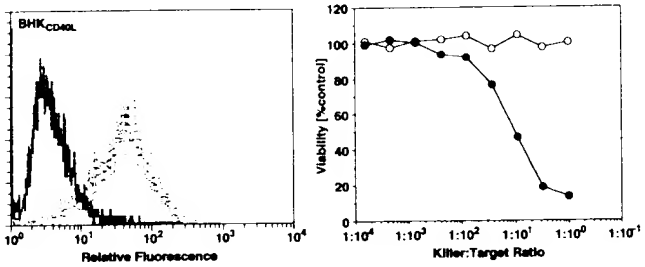

Figure 5. CD40L-expressing cells are cytotoxic on hybrid receptor expressing target cells. BHK_{CD40L} cells were immunostained with rabbit anti-CD40L (·····) or rabbit anti-CD14 Ab as a control (——) and FITC-conjugated goat anti rabbit $F(ab')_2$Ab. Stained cells were analyzed by FCM (left panel). The bioactivity of the BHK_{CD40L} transfectants was tested on $A9_{CT}$ targets. For this purpose the BHK_{CD40L} cells were fixed with 3 % formaldehyde. After rigid washing they were added to the $A9_{CT}$ (●) or $A9_{mock}$ cells (○) at the indicated killer : target ratios together with CHX (50 µg/ml). Viability of the target cells was determined 16 to 18 h later by the neutral red uptake method.

3.4 CD40/TNFR hybrid activating reagents are less potent in a B cell assay measuring CD40-mediated rescue from apoptosis

For all hybrid-stimulating reagents CD40 activity was determined as the ability to rescue BL41 Burkitt lymphoma cells from ionomycin-induced apoptosis. On BL41 cells CD40 was expressed at a similar level as the chimera on the transfectants (Fig. 6, left panel and Fig. 1B right panels). Surprisingly, only rabbit anti-CD40 Ab and BHK_{CD40L} cells increased the B cell viability, albeit at concentrations that were 16-fold higher than those required for hybrid receptor-mediated killing of A9 cells (Fig. 6, right panel). The best rescue from ionomycin-induced cell death was obtained with BHK_{CD40L} cells. Monoclonal anti-CD40 Ab and the soluble IL-4R/CD40L construct were only marginally active (Fig. 6, right panel), even when tested at 250-fold higher concentrations than those needed to obtain half-maximal killing on $A9_{CT}$ cells (data not shown). These data demonstrate that this assay applying the principle of CD40/p55TNFR-mediated cytotoxicity detects CD40-activating reagents with much higher sensitivity than a conventional CD40 assay in B cells.

3.5 Herpesvirus saimiri-transformed T cells constitutively express active CD40L and kill hybrid-expressing transfectants

Exploiting their superior sensitivity, we used the hybrid transfectants to assay cells naturally expressing CD40L for bioactivity. In screening various human T cell lines we found that two H. saimiri-transformed $CD4^+$ cell lines, Kesting and CB15, constitutively express CD40L at high levels (Fig. 7, left panels). Indeed, CB15 T cells tested on $A9_{CT}$ target cells displayed a marked cytotoxic effect. However, CB15 also killed $A9_{mock}$ targets although fivefold less effectively (data not shown). This result is expected since CB15 cells show the typical phenotype of mature activated T lymphocytes and may produce cytotoxic cyto-

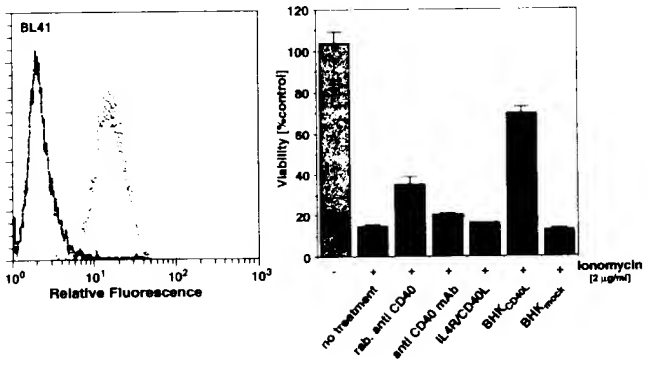

Figure 6. Polyclonal anti-CD40 Ab and cell surface CD40L rescue BL41 B cells from ionomycin-induced apoptosis. To determine CD40 expression (left panel) the BL41 B cells were incubated with anti-CD40 (BB20) (·····) or anti-CD4 mAb (——) as a control, then stained with FITC-conjugated goat anti-mouse $F(ab')_2$Ab and analyzed by FCM. The ability of anti-CD40 Ab, the soluble CD40L construct, BHK_{CD40L} and BHK_{mock} transfectants to rescue BL41 cells from ionomycin-induced cell death was assayed as described in Sect. 2.8. All reagents or cells were used at concentrations 16-fold higher than those required for 50 % cytotoxicity on $A9_{CT}$ targets.

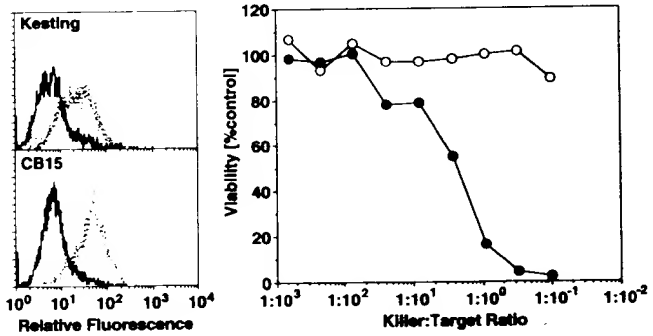

Figure 7. H. saimiri-transformed $CD4^+$ T cells express bioactive CD40L. CD40L expression on cells of the H. saimiri-transformed T cell lines Kesting and CB15 was analyzed by FCM (left panels) after immunostaining with rabbit anti-CD40L (·····) or rabbit anti-CD14 pAb (——) and FITC-conjugated goat anti-rabbit $F(ab')_2$Ab. To assay the bioactivity of cell surface CD40L the CB15 cells were added to BHK_{CT} (●) or BHK_{mock} cells (○) at the indicated killer : target ratios together with CHX (50 µg/ml). After 16 to 18 h, the cells were carefully rinsed with medium to remove all CB15 cells and the viability of the remaining BHK cells was determined by neutral red staining.

kines such as TNF and LT-α. The TNF- and LT-α-resistant BHK_{cT} proved to be more appropriate target cells for the isolated detection of CD40L activity on T cells. CB15 cells killed BHK_{cT} targets with half-maximal effect at a K:T ratio of 1:2 (Fig. 7, right panel, filled symbol). In contrast to the $A9_{mock}$ cells, BHK_{mock} targets showed no significant response to CB15 even at K:T rations of 10:1 (Fig. 7, right panel, open symbol). This is the first evidence that H. saimiri-transformed T cells may constitutively express bioactive CD40L.

4 Discussion

In this study we examined the functional properties of a chimeric receptor composed of CD40 and the p55TNFR. As CD40 and the p55TNFR belong to the same family of proteins [35, 36] we investigated whether their structural relationship also implicates similar activation mechanisms. Based on this hypothesis it should be possible to construct a hybrid receptor whose ligand specificity depends on the respective ECD, whereas the cytoplasmic domain should determine its biological activity.

Here we demonstrate that the CD40/p55TNFR chimera is functionally active. Consistent with the natural function of its cytoplasmic domain the chimera displays strong cytotoxic activity in different cell lines that are susceptible to p55TNFR-mediated cell death (SV80, A9) or like the BHK cells may be rendered sensitive upon transfection with the human p55TNFR [56]. Our data show that large parts of the TNFR including the whole ECD and TMD can be exchanged with CD40 sequences without the loss of cytotoxic function. This underlines not only the close structural relationship between CD40 and the p55TNFR but also indicates that the TNFR ECD and TMD are not essential for its cytotoxic activity.

Interestingly, the ligands of both receptors, CD40L and TNF or LT-α are also members of the same gene family [35, 36]. They show common structural features: (i) CD40L and TNF are type II transmembrane proteins [1, 2, 57]. (ii) A trimeric structure with was demonstrated for TNF and LT-α [58–62] was also postulated for the CD40L [2, 9, 63]. Ligand-mediated trimerization was proposed to be the crucial activation mechanism common to all members of the TNFR family [35, 36, 62]. Thus a functional hybrid receptor should be triggerable by the natural ligand binding to the corresponding ECD.

Indeed, our data demonstrate that the CD40/TNFR hybrid not only delivered a strong cytotoxic signal after cross-linking with polyclonal anti-CD40 Ab, but also with CD40L. Both a recombinant soluble CD40L fusion protein and the membrane-bound form of CD40L were active. These results suggest that TNF and CD40L activate their receptors via the same mechanism, which seems to be aggregation. Since two commercial and four prepared monoclonal anti-CD40 Ab were able to efficiently elicit the cytotoxic response, it appears that dimerization of the CD40/TNFR hybrid is sufficient to trigger TNFR ICD-dependent signals.

In contrast CD40 wild-type activation seemed to require a higher aggregation level. In an assay measuring CD40-mediated B cell rescue from apoptosis, rescue ability correlated with the aggregation capacity of the CD40-stimulating reagents: CD40L-bearing cells were the most potent; polyclonal anti-CD40 Ab displayed an intermediate, soluble CD40L or the mAb, however, only marginal, activity. There are two explanations for this phenomenon: (i) B cell rescue might work according to the threshold principle, thus a minimal signal strength would be necessary to overcome a certain threshold required for survival. (ii) Alternatively, the amplification systems mediating TNFR responses may be more effective than those delivering CD40 signals. Both mechanisms could explain why the chimera detects CD40-stimulating reagents at higher sensitivity than CD40. Generalizing from this result, the principle of fusing the extracellular domain of non-cytotoxic members of the TNFR family to a cell death-mediating ICD, *e.g.* the ICD of the TNFR [64] or fas [65, 66], might be a useful and highly sensitive approach for detecting other non-cytotoxic ligands belonging to the TNF ligand family.

As an application of this principle we used the CD40/TNFR hybrid to study natural CD40L activity on human T cells. Interestingly, we found that the H. saimiri-transformed $CD4^+$ T cell lines CB15 [44] and Kesting [45] constitutively express CD40L on their surface. Keeping in mind that other cytotoxic factors produced by T cells, such as TNF or LT-α [67, 68], might interfere with our cytotoxicity assay, the T cells were tested on BHK transfectants which are highly resistant to human TNF effects. Indeed CB15 cells elicited a strong cytotoxic response on the hybrid receptor but not on mock-transfected BHK cells, revealing the constitutive biological activity of CD40L on H. saimiri virus-transformed $CD4^+$ T cells.

Traditional CD40 assays, such as the measurement of B cell proliferation [16, 18–20], isotype switching [14–17] or B cell rescue from apoptosis [23– 25] either depend on co-stimulatory signals or lack appropriate sensitivity. Our results demonstrate that the CD40/TNFR hybrid not only detects CD40-stimulating reagents with high sensitivity, but also with the required specificity. As shown with the CB15 cells, the hybrid-based assay system allows the isolated detection of CD40L even in the presence of other potent cytokines. Therefore, this system will be useful for the dissection of CD40L-mediated events in T-B cell interactions and for the detection of defective CD40L molecules in putative hyper-IgM syndrome patients.

The authors thank Mrs. Kirstin Gebauer for excellent technical assistance. We are also grateful to Dr. Dolores Schendel for critical review of the manuscript and to Drs. Hans Smola and Georg Honold for helpful discussions. Drs. Helmut Fickenscher and Bernhard Fleckenstein are thanked for the herpesvirus saimiri-transformed T cell lines.

Received September 1, 1994; accepted October 20, 1994.

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TI   Identification of a protein with homology to hsp90 that binds the
     type 1   ***tumor***     ***necrosis***     ***factor***   receptor.
SO   JOURNAL OF BIOLOGICAL CHEMISTRY, (1995 Feb 24) 270 (8) 3574-81.
     Journal code: HIV. ISSN: 0021-9258.
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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 270, No. 8, Issue of February 24, pp. 3574–3581, 1995
Printed in U.S.A.

Identification of a Protein with Homology to hsp90 That Binds the Type 1 Tumor Necrosis Factor Receptor*

(Received for publication, August 15, 1994, and in revised form, November 28, 1994)

Ho Yeong Song, James D. Dunbar, Yuan Xin Zhang, Danqun Guo, and David B. Donner‡

From the Department of Physiology and Biophysics and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202

The yeast-based two hybrid has been used to identify a novel protein that binds to the intracellular domain of the type 1 receptor for tumor necrosis factor (TNFR-1IC). The TNF receptor-associated protein, TRAP-1, shows strong homology to members of the 90-kDa family of heat shock proteins. After *in vitro* transcription/translation and ^{35}S labeling, TRAP-1 was precipitated using a fusion protein consisting of glutathione *S*-transferase and TNFR-1IC, showing that the two proteins directly interact. The ability of deletion mutants of TNFR-1 to interact with TRAP-1 was tested using the two hybrid system. This showed that the amino acid sequences that mediate binding are diffusely distributed outside of the domain in the C terminus of TNFR-1IC that signals cytotoxicity. The 2.4-kilobase TRAP-1 mRNA was variably expressed in skeletal muscle, liver, heart, brain, kidney, pancreas, lung, and placenta. TRAP-1 mRNA was also detected in each of eight different transformed cell lines. Identification of TRAP-1 may be an important step toward defining how TNFR-1, which does not contain protein tyrosine kinase activity, transmits its message to signal transduction pathways.

Tumor necrosis factor (TNF)[1] is produced predominantly by macrophages activated by infections or malignancies (1–3). This potent multifunctional cytokine was first characterized by its ability to induce the hemorrhagic necrosis and regression of cancers in animals and by the cytotoxic response that it can elicit from transformed cells *in vitro*. Subsequent studies have shown that through interactions with virtually every type of cell, TNF also promotes immunity, antiviral responses, inflammation, shock, and metabolic alterations, including cachexia, which accompany disease states (1–3). Such diverse and profoundly important functions have made TNF the subject of intense investigation.

The first step in TNF action is binding to specific receptors that are expressed on essentially all cells (4–7). Two TNF receptors have been identified as proteins of 55-kDa (the type 1 receptor, TNFR-1) and 75-kDa (the type 2 receptor, TNFR-2) (8–11), and their cDNAs have been cloned (12–14). The extracellular domains of the TNF receptors share homologies with one another and with a group of cell surface receptors that include the FAS antigen, the low affinity NGF receptor, the murine cDNA clone 4–1BB from induced helper and cytolytic T cells, the B cell surface antigen CD40, the OX40 antigen of activated CD4-positive rat lymphocytes, and the T2 antigen of the Shope fibroma virus (15). The intracellular domains do not display sequence similarities and couple to different signal transduction pathways. For this reason, the receptors induce distinct responses: TNFR-1 promotes cytotoxicity, fibroblast proliferation, antiviral responses, and the host defense against microorganisms and pathogens (10, 16–19); TNFR-2 plays a role in cytotoxicity that is still being defined (20–22), inhibits early hematopoiesis (23), is involved in the proliferation of monocytes (24), and promotes the proliferation of T cells (25).

Neither TNF receptor contains intrinsic protein tyrosine kinase activity or any recognizable motif (12–14), which suggests a mechanism through which the signal inherent in the hormone-receptor complex can be transmitted to signaling mechanisms. This may suggest that associated proteins, rather than the intracellular domain of either TNF receptor, act as essential elements in signal transmission. Such accessory molecules may be cytoplasmic tyrosine kinases, exemplified by JAK2, which interacts with the erythropoietin and other receptors (26), or non-tyrosine kinases, such as gp130, which associates with the interleukin-6 and other receptors (27). Consistent with the hypothesis that TRAPS may be important to TNF action are recent reports describing co-precipitation of TNFR-1 with a protein kinase activity (28, 29). The present studies were initiated to identify proteins that associate with the intracellular domain of TNFR-1 (TNFR-1IC) and might then initiate signal transduction.

To address this problem, we used the yeast-based two hybrid system, a method for studying protein-protein interactions (30, 31). This method is based on the properties of the yeast GAL4 protein, which consists of separable domains that mediate DNA binding and transcriptional activation. Plasmids encoding two hybrid proteins, one consisting of the GAL4 binding domain (GAL4-BD) fused to protein *X* and the other consisting of the GAL4 activation domain (GAL4-AD) fused to protein *Y*, are cotransformed into yeast. Interaction between these proteins permits transcriptional activation of an integrated copy of the *GAL4-lacZ* reporter gene.

To identify proteins that interact with TNFR-1IC, a plasmid

* This work was supported by a grant from the Indiana Affiliate of the American Diabetes Association (to D. B. D.), by a postdoctoral fellowship from the Indiana Affiliate of the American Heart Association (to H. Y. S.), by a postdoctoral fellowship from the Walther Oncology Center (to Y. X. Z.), and by a predoctoral fellowship from the Indiana Affiliate of the American Heart Association (to D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U12595, U12596.

‡ To whom correspondence should be addressed. Tel.: 317-278-2155; Fax: 317-274-3318.

[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR-1, 55-kDa type 1 tumor necrosis factor receptor; TNFR-2, 75-kDa type 2 tumor necrosis factor receptor; TNFR-1IC, intracellular domain of TNFR-1; TNFR-2IC, intracellular domain of TNFR-2; TRAP-1, TNF receptor-associated protein-1; TRAP-2, TNF receptor-associated protein-2; GAL4-BD, the GAL4 binding domain; GAL4-AD, the GAL4 transactivation domain; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; TnT, troponin T; PCR, polymerase chain reaction; kb, kilobase(s); RACE, rapid amplification of cDNA ends.

in which TNFR-1IC was fused with GAL4-BD (pGBT-TNFR1IC) was cotransformed into yeast together with a pool of plasmids encoding GAL4-AD/HeLa S3 cDNA library fusion proteins. This has led to identification of a gene encoding a novel protein that binds TNFR-1IC. The protein was precipitated using a fusion protein consisting of glutathione *S*-transferase and TNFR-1IC, demonstrating that it interacts directly with the TNF receptor. The protein shows significant homology to members of the 90-kDa family of heat shock proteins (hsp90) and is widely expressed in normal tissues and transformed cells. We also used the two hybrid system to assay the ability of deletion mutations of TNFR-1IC to interact with the newly discovered protein. These experiments show that the amino acid sequences necessary for interaction are diffusely distributed outside of the cytotoxicity domain in TNFR-1IC. Identification of this TNF receptor-associated protein, which we call TRAP-1, is an important step toward defining how TNFR-1 couples to signal transduction pathways and transduces TNF binding into responses.

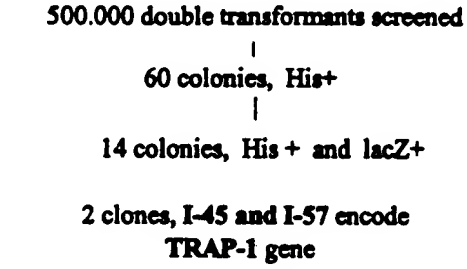
MATERIALS AND METHODS

Bacterial and Yeast Strains—Yeast strains for two hybrid experiments were obtained from Clontech as components of the Matchmaker Two Hybrid System. Yeast strains SFY526 (*MATa, ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu 2–3, 112, can^r, gal4–542, gal80–538, URA3::GAL1-lacZ*) and HF7c (*MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112 gal4–542, gal80–538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ*) were used to assay for protein-protein interactions and for library screening, respectively. SFY526 has the upstream-activating sequence and TATA sequences of the *GAL1* promoter fused to the *lacZ* gene. In HF7c, *HIS3* is fused to a *GAL1* promoter sequence and *LacZ* is fused to 3 copies of a 17-mer *GAL4* consensus sequence plus the TATA sequence of the *CYC1* promoter. The *Escherichia coli* strain of XL1-blue (Stratagene) was employed in the cloning of plasmids unless otherwise noted.

DNA Manipulation—Yeast shuttle vectors pGBT9 (GAL4-BD), pGAD424 (GAL4-AD), pLAM5′ (the lamin gene), pVA3 (the p53 gene), and pTD1 (the SV40 large T antigen gene) were from Clontech. PCR subcloning was used to amplify and insert TNFR-1IC into the unique *Eco*RI and *Bam*HI of pGBT9 (pGBT-TNFR1IC) using a cDNA clone of TNFR-1 in pUC19 (a gift from Dr. H. Loetscher, Hofmann-LaRoche Inc., Geneva, Switzerland) as the template. The intracellular domains of TNFR-2 (TNFR-2IC) (a gift from Dr. H. Loetscher) and the human FAS antigen (a gift from Dr. S. Nagata, Osaka Bioscience Insitute, Osaka, Japan) (FAS-IC) were amplified and subcloned into pGBT9 as described for TNFR-1IC. PCR products were run on a low melting point gel, cut out, melted, cleaned using a DNA cleanup kit (Promega), digested with appropriate enzymes, and finally ligated to the appropriate vector. Plasmid isolation was accomplished using the Wizard Miniprep and Maxiprep kits from Promega.

Six primers were designed to amplify various regions within TNFR-1IC to make a series of deletion mutants. For oriented cloning, the 5′ primers were linked to *Eco*RI, and the 3′ primers were linked to *Bam*HI. All constructs were sequenced at the fusion sites to confirm in frame fusion of TNFR-1IC and TNFR-1IC deletion mutants with GAL4-BD.

a



b

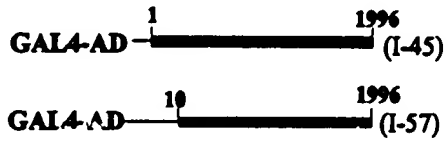


FIG. 1. *a*, two hybrid library screen. pGBT9-TNFR1IC was used to screen a HeLa S3 cDNA library. Among the interacting clones, I-45 and I-57 encode partial sequences of TRAP-1 gene. *b*, schematic of two clones. Clone I-45 contains the partial TRAP-1 gene that spans 1996 bases up to the beginning of the poly(A) tail, which is in frame with GAL4-AD. Clone I-57 is shorter by 9 bases, initiating at base 10. *c*, specificity of interactions. Yeast strains transformed with combinations of plasmids fused to the GAL4-BD and with clone I-45 were assayed for activation of β-galactosidase. The p53 gene (pVA3) and the SV40 large T antigen (pTD1) associate strongly and serve as a positive control. pGBT-FAS is a GAL4-BD/FAS-IC fusion; pGBT-TNFR2IC is a GAL4-BD/TNFR-2IC fusion.

c

DNA binding	Activating	beta-galactosidase activity	filter color
—	I-45	0.6 ± 0.5	white
pGBT-9	I-45	0.3 ± 0.2	white
pGBT-TNFR1 IC	I-45	116 ± 2.5	blue
pLAM5	I-45	0.4 ± 0.3	white
pGBT-FAS	I-45	0.6 ± 0.0	white
pGBT-TNFR2 IC	I-45	0.7 ± 0.0	white
pVA3	pTD1	494 ± 70	blue

Two Hybrid Library Screening—The HeLa S3 Matchmaker cDNA library was purchased from Clontech. pGBT9-TNFR1IC was transformed into HF7c using the lithium acetate procedure. After determining that TNFR-1IC alone does not contain any latent transcriptional activity in HF7c, the transformant was grown overnight in Trp synthetic medium, to ensure that every cell contained pGBT9-TNFR1IC, and sequentially transformed with 500 μg of the HeLa S3 Matchmaker cDNA (prepared in the two hybrid activation vector pGADGH). Double transformed cells on 50 Leu , Trp , His plates were incubated for 5 days at 30 °C before positive colonies were picked, restreaked onto triple minus plates, and assayed for the *lacZ* phenotype. Library clones that activated the *lacZ* reporter gene only in the presence of pGBT9-TNFR1IC were chosen for sequencing, which was conducted using the Sequenase sequencing kit (U. S. Biochemical).

Color Development Assays—Yeast transformants were assayed for β-galactosidase activity using filter and liquid assays. For filter assays, transformants were transferred to nitrocellulose filters, permeabilized in liquid nitrogen, and placed on Whatman No. 1 filter paper that had been soaked in Z buffer (60 mM Na_2HPO4, 40 mM NaH_2PO4, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol) containing 1.0 mg/ml 5 bromo-4-chloro-3-indolyl β-D-galactoside at 30 °C. Color developed between 5 min and 10 h. For the liquid assay, cells were diluted 5-fold in rich media (YPD), grown to mid-log phase (A_{600}, 0.4–0.8), snap frozen in liquid nitrogen, thawed at 37 °C, and further disrupted by vortexing with glass beads. The procedure of Miller (32) was then used to quantitate β-galactosidase activity; however, chlorophenyl-red-β-D-glactopyranoside (Boehringer Mannheim) was used for color development, and cell pellets were resuspended in 900 μl of 100 mM HEPES, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 1% bovine serum albumin. 100 μl of 50 mM chlorophenyl-red-β-D-glactopyranoside was added following cellular disruption, and the amount of liberated chlorophenyl-red-β-D-glactopyranoside was determined by A_{574}. Numbers represent β-galactosidase activity in Miller units and are expressed as the mean of triplicate determinations ± S.D.

5′-RACE PCR and cDNA Screen—The 2-kb cDNA insert of clone I-45 from the two hybrid library screen was sequenced, and the restriction sites were mapped. PCR was performed to amplify the sequence missing at the 5′-end of the TRAP-1 gene using 5′-RACE Ready cDNA from human liver (Clontech) as a template. Two TRAP-1 gene-specific primers (GSP1, GSP2) were designed based on the TRAP-1 partial sequence of I-45 that binds near the N terminus of the known sequence. The sequences of the gene-specific primers are 5′-CTTTGTCTCGGCCTGGAAC-3′ (GSP1) and 5′-CGGGATCCCATGTTTGGAAGTGGAACCCT-3′ (GSP2). Primary and secondary PCR was performed according to the protocol provided by Clontech.

Using the N terminal 0.6-kb PCR product of the TRAP-1 gene from I-45 as a probe, a HeLa S3 cDNA library in lambda ZAPII (Stratagene) was screened using standard techniques. The longest 2.2-kb insert was rescued as a phagemid (pBluescript-TRAP1) by coinfection with the helper phage.

Northern Blotting—Membranes pre-blotted with poly(A)$^+$ RNA from a variety of human tissues and cancer cell lines were obtained from Clontech. A 0.6-kb N-terminal PCR product of clone I-45 and an 0.8-kb probe to hsp90-β (Stressgen Biotech. Corp.), which were labeled using a Prime-A gene labeling kit from Promega, were used as probes. Hybridization, washing, and stripping of the blots were conducted according to instructions provided by Clontech.

Preparation of GST-TNFR-1IC Fusion Proteins—Three GST-TNFR1IC fusion constructs were prepared by amplifying and inserting desired portions of TNFR-1IC into the unique *Eco*RI and *Bam*HI sites of pGEX-2T (Pharmacia Biotech. Inc.): 1) full-length TNFR-1IC (amino acids 205–413, GST-TNFR1IC); 2) part of the N-terminal half of TNFR-1IC (amino acids 243–315, GST-TNFR1NH$_2$); and 3) the C-terminal half of TNFR-1IC (amino acids 316–413, GST-TNFR1COOH). After induction, cells were grown at 30 °C for 3 h, suspended in lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1% 2-mercaptoethanol), and sonicated. The lysate was centrifuged (12,000 rpm, 30 min), and one-tenth volume of 50% GST-agarose slurry (Sigma) was incubated with the supernatant for 1 h at 4 °C. The beads were washed three times with lysis, buffer and the purity of the GST fusion proteins was confirmed by SDS-PAGE.

In Vitro Translation—The TnT-coupled rabbit reticulocyte lysate system (Promega) was used for one tube transcription/translation according to the instructions of the manufacturer. Rabbit reticulocyte was mixed with 1 μg of pBluscript-TRAP1 and T3 RNA polymerase, after which amino acid mixture without methionine plus [^{35}S]methionine was added to the incubate. After 2 h at 30 °C, expression of TRAP-1 was characterized by SDS-PAGE and molecular image analysis (Bio-Rad). *In vitro* translation yielded a dominant 65-kDa protein, which is the predicted size assuming that the first internal Met codon of the pBluescript-TRAP1 is the start site for most translation.

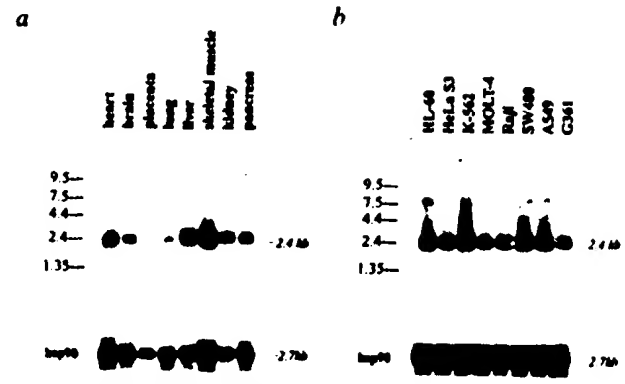


FIG. 2. **Expression of TRAP-1 and human hsp90-β.** Pre-blotted membranes containing mRNAs from human tissues (*a*, *left*) or cancer cell lines (*b*, *right*) were probed with a 0.6-kb random primed N-terminal PCR product of the TRAP-1 gene in I-45 (*top*) and a specific probe for human hsp90-β (*bottom*). The cancer cell lines from *right* to *left* were HL-60 promyelocytic leukemia cells, HeLa S3 cells, k-562 chronic myelogenous leukemic cells, MOLT-4 lymphoblastic leukemia cells, Burkitt's lymphoma Raji, SW480 colorectal adenocarcinoma cells, A549 lung carcinoma cells, and G361 melanoma cells. *Numbers* to the *left* of the blots indicate relative size in kb. The *line* to the *right* of the *top panel* indicates the 2.4-kb TRAP-1 mRNA. The *line* to the *right* of the *bottom panel* indicates the 2.7-kb hsp90-β mRNA.

In Vitro Binding—Agarose-bound GST alone (10 μg), GST-TNFR1IC (10 μg), GST-TNFR1NH$_2$ (30 μg), or GST-TNFR1COOH (10 μg) was added to binding buffer (20 mM Tris, pH 7.7, 0.5% Nonidet P-40, 200 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1% 2-mercaptoethanol) and 40 μl of reticulocyte lysate containing [^{35}S]methionine-labeled TRAP-1. After 1 h at 4 °C, the agarose beads were washed four times with binding buffer before addition of SDS-PAGE sample buffer, electrophoresis, and phosphoimage analysis.

Two Hybrid Mapping of the TRAP-1 Binding Site in TNFR-1IC—TNFR-1IC truncations spanning amino acids 205–280, 205–359, 278–359, 278–426, and 352–426 were fused in frame with the GAL4-BD by PCR subcloning. These constructs and I-45 were cotransformed into SFY526, which were assayed for the *lacZ* phenotype. Expression of the GAL4-BD/TNFR-1IC fusion proteins was determined by Western blotting using a polyclonal antibody directed against GAL4-BD (Upstate Biotechnology, Inc.). Briefly, yeast transformants were grown overnight in minimal media and then diluted 5-fold in YPD broth. At mid-log phase, cells were harvested by centrifugation, washed twice with ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), resuspended in TE buffer containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin, and lysed by agitation with glass beads. Supernatants were fractionated by SDS-PAGE and transferred to Immobilon-P, which was hybridized with the GAL4-BD antibody at 1:2000 dilution and detected by ECL (Amersham Corp.).

RESULTS

To identify proteins that interact with TNFR-1IC, plasmids encoding TNFR-1IC fused with the GAL4-BD (pGBT-TNFR1IC) and HeLa S3 Matchmaker cDNA were cotransformed into HF7c. Double transformant colonies were screened and selected for histidine prototrophy (Fig. 1*a*). Colonies isolated as His$^+$ transformants were assayed for *lacZ* expression to eliminate false positives, an effective procedure as the *His3* and *lacZ* reporter genes in HF7c are under control of dissimilar promoters. Activation domain library plasmids encoding potential TRAPs were isolated from the double positive colonies, and the GAL4-AD/TRAP gene fusion site was sequenced. We found two groups of cDNAs that encode distinct proteins: TNF receptor-associated protein 1 (TRAP-1) and a second receptor associated protein (TRAP-2), which will be described elsewhere.

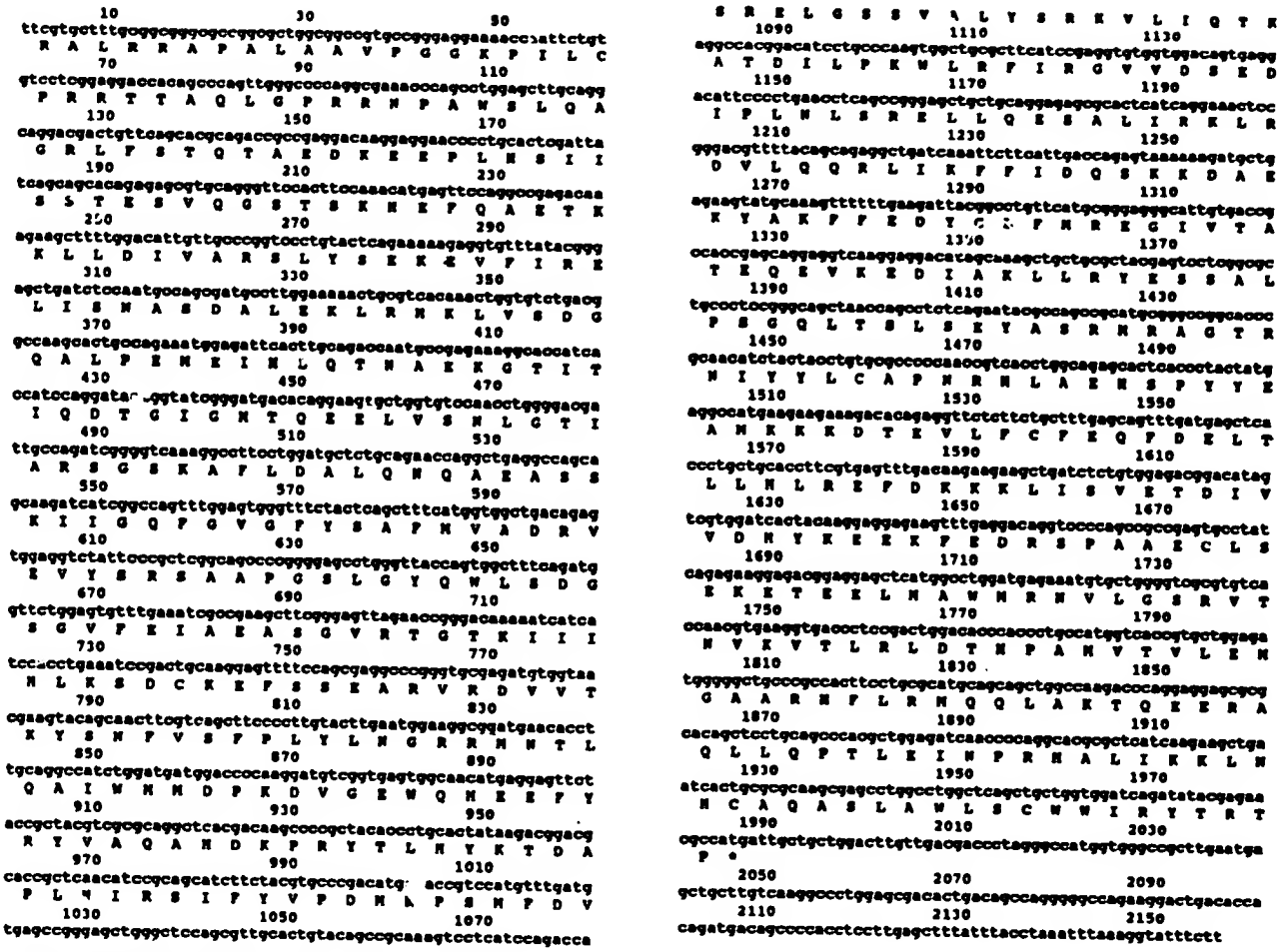


FIG. 3. **Sequence of TRAP-1 cDNA with predicted amino acid sequence.** Clone I-45, which includes the poly(A) tail, and a cDNA segment obtained from 5′-RACE PCR were sequenced. The sequence spans 2156 bases without the poly(A) tail and encodes a protein of 661 amino acids.

Two TRAP-1 clones, I-45 and I-57, were sequenced at the fusion site; both were in frame with GAL4-AD, and I-57 was shorter than I-45 by 9 bases (Fig. 1*b*). Sequence analysis of the cDNA insert of I-45 showed that the partial TRAP-1 gene spans 1996 bases with an open reading frame ending at base 1827. From the fusion site, this clone encodes a protein of 608 amino acids.

Experiments were conducted to establish that interaction of TNFR-1IC with TRAP-1 is specific (Fig. 1*c*). I-45 by itself was incapable of activating β-galactosidase activity, which shows that TRAP-1 does not contain a latent transcriptional activator. GAL4-BD alone, lamin, TNFR-2IC, or FAS-IC, which contains a cytotoxicity domain with homology to that found in TNFR-1IC, did not interact with TRAP-1. Only when the TNFR-1IC gene was cotransformed with I-45 was activation of the *lacZ* reporter gene detected. These observations rule out the possibility that TRAP-1 nonspecifically interacts with other proteins.

To identify the full-length size and distribution of expression of TRAP-1 RNA, we used a 0.6-kb N-terminal PCR product of I-45 as a probe to hybridize blots of multiple normal tissues and various transformed cell lines. As shown in Fig. 2, there was a single TRAP-1 transcript of about 2.4 kb. To obtain the full-length cDNA for TRAP-1, we used 5′-RACE PCR and also screened a lambda ZAPII cDNA library. The 5′-RACE PCR extended the known sequence of TRAP-1 by 180 bases. The combined sequence of I-45 with the 5′-RACE PCR product spans 2.2 kb (*versus* 2.4 kb, mRNA size) and is predicted to encode a protein of 661 amino acids (Fig. 3). A highly compressed GC-rich region of about 30 bases at the 5′-end of the PCR product may have prevented the reverse transcriptase from reading to the 5′-end of the message. Similarly, the longest phage clone isolated as a phagemid from the lambda ZAPII library search was 2.2 kb (pBluescript-TRAP1). However, it is most important that the sequence defined by the two hybrid screen and the 5′-RACE PCR encodes virtually the complete coding sequence of TRAP-1 and contains all of the elements necessary for binding to TNFR-1IC.

To identify homologous proteins, the predicted amino acid sequence of TRAP-1 was used to search the National Center for Biotechnology Information (NCBI) data base using Blast command. TRAP-1 shares significant homology, 34% sequence identity and overall homology of about 60% when conserved substitutions are included, with members of the hsp90 family (33) (Fig. 4). Homologies between hsp90s and TRAP-1 are not restricted to a single sequence of amino acids but reside in at least six distinct domains. A striking difference between hsp90s and TRAP-1 is the absence of a highly charged domain in the latter, which is present in the former.

Northern blot analysis (Fig. 2, *top*) showed that TRAP-1 mRNA is variably present in eight different normal tissues and was in eight transformed cell lines. To rule out the possibility that the probe used to define the size and tissue distribution of TRAP-1 mRNA nonspecifically recognizes hsp90, the blots used for the experiments in Fig. 2 were stripped and hybridized with

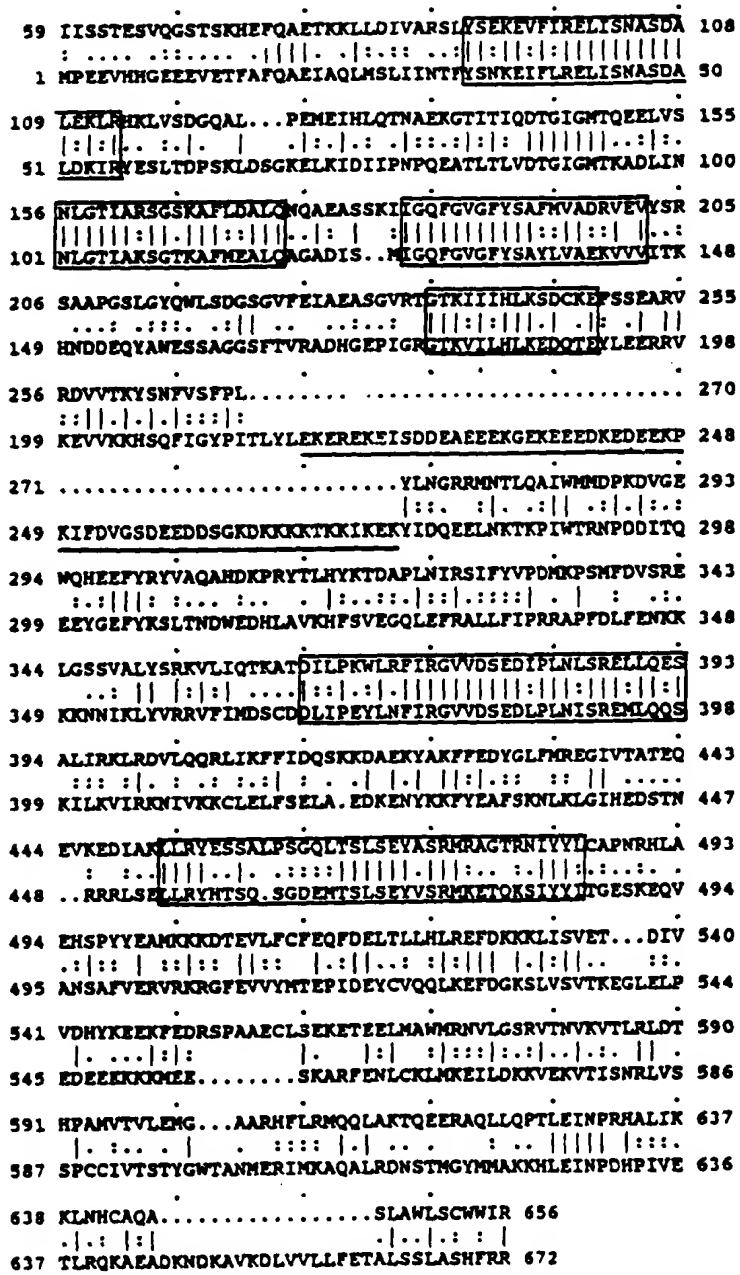


FIG. 4. **Amino acid sequence alignment of rat hsp90-β with TRAP-1.** Sequence alignment was obtained using Bestfit command in Genetics Computer Group software . The *upper sequence* is TRAP-1, and the *lower sequence* is rat hsp90. *Straight lines* between amino acids indicate identity, and *dotted lines* indicate conserved substitutions. The highly charged segment in rat hsp90 is *underlined*, while obviously conserved domains in TRAP-1 and hsp90 are *boxed*.

a probe to hsp90 (Fig. 2, *bottom*). The hsp90 mRNA was distinguished from that of TRAP-1 by size (2.4 compared with 2.7 kb, respectively) and relative expression level in normal tissues and transformed cells.

An *in vitro* binding assay was developed to demonstrate direct interaction between TRAP-1 and TNFR-1IC and to establish that identification of TRAP-1 as a TNF receptor-associated protein was not an artifact of the two hybrid system. TRAP-1 expressed by *in vitro* translation and labeled with [^{35}S]methionine was incubated with agarose GST, and with GST fusion proteins containing full-length TNFR-1IC (GST-TNFR1IC), the N-terminal half of TNFR-1IC (GST-TNFR1NH$_2$) and the C-terminal half of TNFR-1IC (GST-TNFR1COOH) (Fig. 5). GST-TNFR1IC and GST-TNFR1NH$_2$ precipitated TRAP-1, whereas GST or GST-TNFR1COOH did not. These observations show that TNFR-1IC binds TRAP-1 without the intermediacy of third proteins and does so in a mammalian cell-free system as well as in yeast. Thus, identification of TRAP-1 as a TNF receptor-associated protein cannot be an artifact associated with the use of the two hybrid system. These observations additionally show that the binding site for TRAP-1 resides in the N-terminal half of TNFR-1IC.

To further define the amino acid sequences important for interaction of TNFR-1IC with TRAP-1, we constructed C- and N-terminal truncations of TNFR-1IC. The deletion mutants were subcloned into the pGBT9 and cotransformed into SFY526 together with I-45 to assay their interactions using the two hybrid system. The relationship of these mutants to TNFR-1IC and their ability to interact with TRAP-1 and thereby activate β-galactosidase is shown in Fig. 6*a*. The results obtained do not directly reflect the affinity of the interaction between TNFR-1IC domains with TRAP-1 since expression of

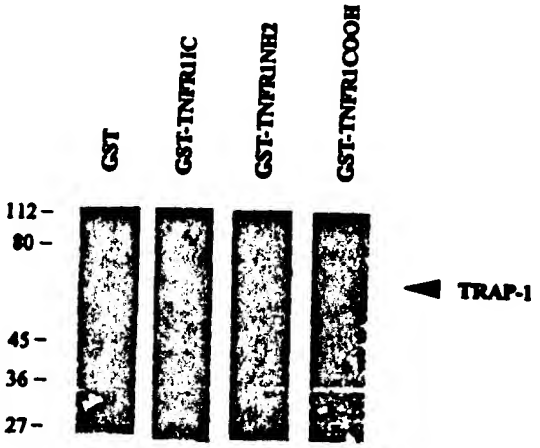
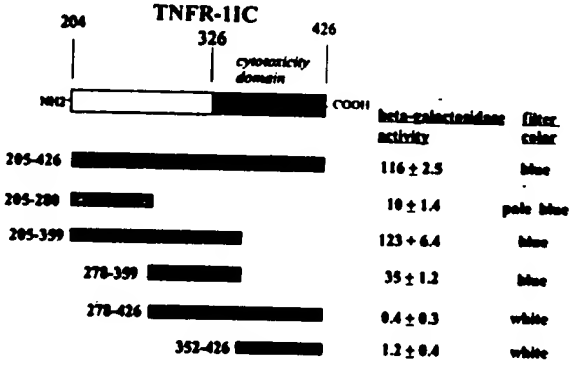


FIG. 5. ***In vitro* precipitation of TRAP-1.** *In vitro* translated and [^{35}S]methionine-labeled TRAP-1 was precipitated using GST fusions of TNFR-1IC. *Lane 1*, GST alone, control; *lane 2*, GST-TNFR1IC; *lane 3*, GST-TNFR1NH$_2$; *lane 4*, GST-TNFR1COOH. The *arrow* indicates TRAP-1 precipitated by GST-TNFR1IC and GST-TNFR1ICOOH.

a



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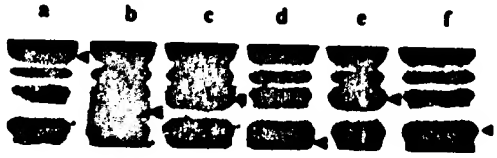

FIG. 6. **Mapping the TRAP-1 binding sites in TNFR-1IC.** *a*, plasmids encoding GAL4-BD/TNFR-1IC deletion mutants were cotransformed with I-45, and β-galactosidase activity was assayed. The *top bar* illustrates the structure of TNFR-1IC. The *blackened horizontal bars* represent the relative size and location of each deletion mutant in TNFR-1IC. *b*, expression of GAL4-BD/TNFR-1IC deletion mutants was assayed by Western blotting using antisera directed to GAL4-BD. *Lane a*, full-length TNFR-1IC (amino acids 205–426); *lane b*, TNFR-1IC amino acids 205–280; *lane c*, TNFR-1IC amino acids 205–359; *lane d*, TNFR-1IC amino acids 278–359; *lane e*, TNFR-1IC amino acids 278–426; *lane f*, TNFR-1IC amino acids 352–426, which consistently appeared to overlap a nonspecific band. The *arrow* to the *right* of each *lane* in the Western blot indicates the position of the fusion protein.

the GAL4-BD/TNFR-1IC fusion proteins in yeast, assayed by Western blotting with an antibody directed against the GAL4-BD (Fig. 6*b*), was somewhat variable. However, the results can be used to determine whether any receptor domain contains recognition elements essential to the interaction between TNFR-1IC and TRAP-1, especially when interpreted together with observations made using GST-TNFR1IC fusion proteins (Fig. 5). Peptides encompassing amino acids 205–280 and 278–359 activated β-galactosidase, showing that the elements that promote interaction of TNFR-1IC with TRAP-1 are diffusely distributed. The avidity with which amino acids 205–359 bound TRAP-1 (123 Miller units of β-galactosidase activity) was more than predicted based on an additive response to amino acids 205–280 and 278–359 (45 Miller units), suggesting that the conformation of the peptide and perhaps the accessibility of crucial binding domains plays a role in regulating interaction. The inability of residues 352–426 to produce β-galactosidase activity shows that the binding sites reside largely, if not entirely, outside of the cytotoxicity domain, a result consistent with the ability of GST-TNFR1NH$_2$ but not GST-TNFR1COOH to precipitate TRAP-1 (Fig. 5). A larger C-terminal peptide (amino acids 278–426) was also unable to activate β-galactosidase, despite the capacity of the peptide encompassing residues 278–359 to do so. One explanation for this result is that the cytotoxicity domain negatively regulates interaction of TNFR-1IC with TRAP-1.

DISCUSSION

Studies taking advantage of the strong species specificity of TNFR-2 for murine compared with human TNF (17) or employing agonist antibodies specific to either TNF receptor (10, 16–20) have shown that signaling through TNFR-1 promotes growth inhibition and cytotoxicity in transformed cells, antiviral activity, proliferation of fibroblasts, induction of NF-kB, accumulation of c-Fos, interleukin-6, and manganous superoxide dismutase mRNAs, prostaglandin E$_2$ synthesis, and HLA class I and II cell surface antigen expression (1–3, 10, 16–20). Mice deficient in TNFR-1 are severely impaired in the ability to clear the bacterial pathogen Listeria monocytogenes and die rapidly from infections; however, these animals are resistant to lipopolysaccharide-mediated septic shock (19). Thus, TNFR-1 plays a decisive role in the host's defense against microorganisms and their pathogenic factors. The significant role of this receptor in TNF action led us to initiate our search for TRAPs using TNFR-1IC as bait in a two hybrid screen of a cDNA library.

The two hybrid system detects proteins capable of interacting with a known protein by transcriptional activation of a reporter gene (30, 31). This method has demonstrated interactions between Bcl-2 and R-*ras* p23 (34), Sos 1 and GRB2 (35), complex formation between Ras and Raf and other protein kinases (36), binding of the human immunodeficiency virus type 1 GAP protein with cyclophilins A and B (37), and also identified proteins that interact with the type 1 receptor for TGF-β (38) and the type 2 receptor for TNF (39). Previously, we used the two hybrid system to demonstrate self-association of TNFR-1IC (40). Evidence for such aggregation was obtained from a screen of a HeLa S3 cDNA library using TNFR-1IC as bait; this yielded a clone encoding a protein encompassing the death domain at the C-terminal of TNFR-1IC (41). Aggregation is independently suggested by the ability of non-functional TNFR-1 deletion mutants to suppress signaling by non-defective endogenous TNF receptors (42). Thus, our previous studies (40) demonstrated that the two hybrid system can successfully identify proteins that interact with TNFR-1, one of which is TNFR-1IC itself.

In this and another study that describes a second TNF receptor-associated protein, TRAP-2,[2] the two hybrid system was used to search for additional proteins that bind TNFR-1IC, leading to the discovery of TRAP-1 and TRAP-2. Both proteins interact specifically with TNFR-1 and not with unrelated pro-

[2] Song, H. Y., Dunbar, J. D. Zhang, Y. X., Guo, D., and Donner, D. B., submitted for publication.

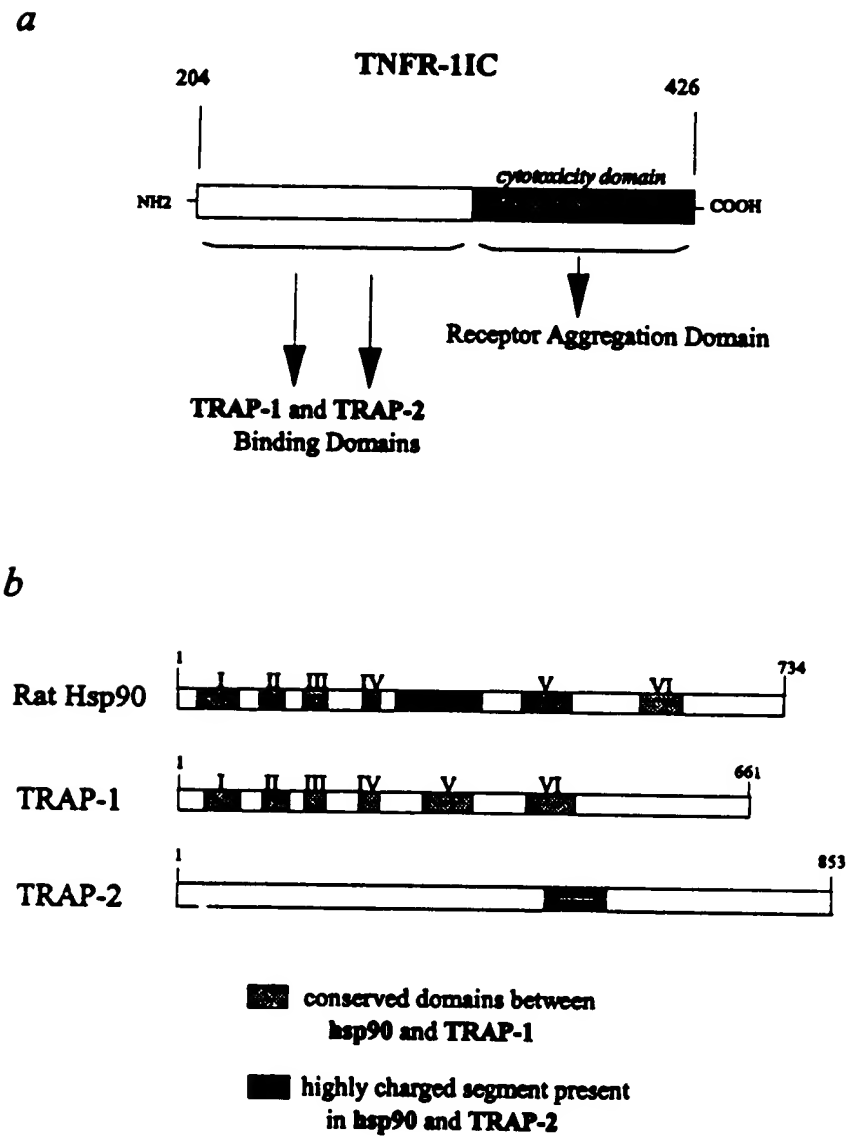

FIG. 7. *a*, map of TRAP binding sites in TNFR-1IC defined using the two hybrid system and by *in vitro* precipitation. *b*, relationship of hsp90, TRAP-1, and TRAP-2. Six domains that share homology in hsp90 and TRAP-1 are indicated by *speckled boxes* and *Roman numerals*. The *black boxes* delineate a highly charged segment in hsp90 and TRAP-2. The length of each *bar* represents the relative size of each protein.

teins. mRNAs for both TRAPs are highly expressed in numerous transformed cell lines and are detected at different levels of expression in the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

Fig. 7*a* summarizes the domain structure of TNFR-1IC, showing the relationships of the death/aggregation domain to the binding sites for TRAP-1 and TRAP-2. Each TRAP binds to sites in TNFR-1IC that reside outside of the death (41, 42) and/or aggregation (40) domain. Mutations in this domain disrupt the ability of TNFR-1 to signal not only cytotoxicity but antiviral activity and induction of nitric oxide synthase as well. These observations should not lead one to conclude that TRAP-1 and TRAP-2 are not involved in TNF action. While the cytotoxicity domain is undoubtedly important for induction of some responses mediated by TNFR-1, it is not the only domain involved in signaling. Recent observations show that the ability of TNF to activate an endosomal acidic sphingomyelinase is abrogated by C-terminal deletions encompassing all or part of the cytotoxicity domain in TNFR-1, whereas activation of a membrane-associated neutral sphingomyelinase is unaffected (43). Ceramide produced by the neutral sphingomyelinase is important for activation of a proline-directed serine threonine kinase and phospholipase A_2. The acidic sphingomyelinase activates NF-kB. Thus, different domains in TNFR-1IC control important and distinguishable second messenger pathways and cellular responses.

TRAP-1 shows strong homology to members of the hsp90 family. Fig. 4 illustrates the presence of at least six domains of high amino acid identity and conservation that relate TRAP-1 and hsp90s. Interestingly, a well conserved, highly charged sequence of amino acids in hsp90 is absent from TRAP-1 but identified in TRAP-2. Fig. 7*b* illustrates the structural relationship of hsp90 to TRAP-1 and TRAP-2. While the significance of the relationship of the TRAPS to one another and hsp90 is not presently known, a basis exists for believing that such stress proteins are important to defining cellular responses to TNF. First, induction of the heat shock response renders transformed cells resistant to TNF and macrophage-mediated cytotoxicity (44). Second, transfection of hsp70s into otherwise responsive cells induces a state of resistance (45), and TNF-induced phosphorylation of hsp28 stress proteins is also associated with protection against cytotoxicity (46). Third, the elements in TNFR-1IC necessary for binding to TRAP-1 and TRAP-2 reside outside of the cytotoxicity/aggregation domain. Large deletions outside of this domain (41), where TRAP-1 and TRAP-2 bind, do not impair the ability of mutant receptors to

induce cytotoxicity or antiviral activity.

Heat shock proteins are likely candidates for service as TRAPs as they act as cofactors or chaperones in cell growth-associated processes, protein folding and transport, and cell division and membrane function (47). Hsp90s associate with steroid receptors, which are thereby stabilized in a non-DNA binding conformation, and also with protein kinases (47). Hsp90s also modulate signaling through transduction cascades. Mutations in a member of the hsp90 family, hsp83, impairs signaling by the sevenless receptor tyrosine kinase, which is required for differentiation of the R7 photoreceptor neuron in *Drosophila* (48). Also, lethal expression of vSrc in *Saccharomyces cerevisiae* is suppressed by a reduction in the level of Hsp82 (49). The diverse properties of hsps suggest that the discovery of TRAP-1, and also TRAP-2, is important and likely to provide insight into the mechanistic basis for TNF action.

Acknowledgment—We gratefully acknowledge the assistance of Dr. Li Zhu (Clontech) in the use of the two hybrid system.

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AU   Bigda J; Beletsky I; Brakebusch C; Varfolomeev Y; Engelmann H; Bigda
     J; Holtmann H; Wallach D
TI   Dual role of the p75   ***tumor***     ***necrosis***
       ***factor***   (  ***TNF***  ) receptor in   ***TNF***
     cytotoxicity.
SO   JOURNAL OF EXPERIMENTAL MEDICINE, (1994 Aug 1) 180 (2) 445-60.
     Journal code: I2V. ISSN: 0022-1007.
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Dual Role of the p75 Tumor Necrosis Factor (TNF) Receptor in TNF Cytotoxicity

By Jacek Bigda, Igor Beletsky, Cord Brakebusch, Yevgeny Varfolomeev, Hartmut Engelmann, Justina Bigda, Helmut Holtmann,* and David Wallach

*From the Department of Membrane Research and Biophysics, The Weizmann Institute of Science, 76100 Rehovot, Israel; and the *Department of Molecular Pharmacology, School of Medicine, 3000 Hannover 1, Germany*

Summary

Whereas there is ample evidence for involvement of the p55 tumor necrosis factor (TNF) receptor (p55-R) in the cytocidal effect of TNF, the role of the p75 TNF receptor (p75-R) in this effect is a matter of debate. In this study, we probed the function of p75-R in cells sensitive to the cytotoxicity of TNF using a wide panel of antibodies (Abs) against the receptor's extracellular domain. Two distinct Ab effects were observed. The Abs triggered signaling for cytotoxicity. This effect: (*a*) was correlated with the extent of p75-R expression by the cells; (*b*) was dependent on receptor cross-linking by the Abs; (*c*) occurred in HeLa cells, but not in A9 cells transfected with human p75-R or in HeLa cells expressing cytoplasmically truncated p75-R mutants, indicating that it involves cell-specific activities of the intracellular domain of the receptor; (*d*) was synergistic with the cytocidal effect of Abs against p55-R. Moreover, it seemed to reverse induced desensitization to the cytocidal effect of anti p55-R Abs, suggesting that it involves mechanisms different from those of the signaling by the p55 TNF-R. In addition, the Abs affected the response to TNF in a way that does not involve the signaling activity of p75-R. These effects: (*a*) could be observed also in cells in which only p55-R signaled for the cytocidal effect; (*b*) were not dependent on receptor cross-linking by the Abs; (*c*) varied according to the site at which the Abs bound to the receptor; and (*d*) were correlated inversely with the effects of the Abs on TNF binding to p75-R. That is, Abs binding to the membrane-distal part of the receptor's extracellular domain displaced TNF from the p75 receptor and enhanced cytocidal effect, whereas Abs that bind to the membrane-proximal part of the extracellular domain — a region at which a conformational change seems to take place upon TNF binding — decreased the dissociation of TNF from p75-R and inhibited its cytocidal effect. The above findings suggest that p75-R contributes to the cytocidal effect of TNF both by its own signaling and by regulating the access of TNF to p55-R.

TNF initiates its multiple effects on cell function by binding to two distinct cell surface receptors (1–8). These two receptor species (p55-R[1] and p75-Rs) are expressed in many cell types, in various amounts and proportions. Both also exist in soluble forms, which are derived proteolytically from the extracellular domain of the corresponding cell surface form (2, 9–11). The soluble TNF receptors (TNF-R) bind TNF in a reversible manner and can thus affect its access to the cell-surface receptors as well as its stability (12).

There is significant sequence homology between the extracellular domains of the two receptors. Both contain a four-fold repetition of a sequence module characterized by conserved location of several (usually six) cysteine residues, as well as of some other amino acid residues (4–8). Homologous repetitive cysteine-rich modules occur in the extracellular domains of a number of other receptors (the TNF/nerve growth factor [NGF] receptor family), including the low-affinity NGF receptor (13), the B cell antigen CD40 (14), the lymphoid activation antigen OX40 (15), the T cell antigens 4-1BB (16) and CD27 (17), the CD30 antigen (18), the Fas/APO-1 receptor (19, 20), and the T2 pox virus gene product (21). Significant structural homology among the

[1] *Abbreviations used in this paper:* A9p75WT, A9 cells expressing transfected wild type p75 TNF receptor; A9 p75CT, A9 cells expressing transfected cytoplasmically truncated p75 TNF receptor; ABTS, 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); CHI, cycloheximide; HeLa p75WT, HeLa cells expressing transfected wild-type p75 TNF receptor; HeLa p75CT, HeLa cells expressing transfected cytoplasmically truncated p75 TNF receptor; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, maltose binding protein; NGF, nerve growth factor, p55-R, p55 TNF receptor; p75-R, p75 TNF receptor; RIPA, Tris-HCl, NaCl, NP-40, deoxycholate, SDS, EDTA; TNF-R, TNF receptor.

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ligands for several of these receptors suggests that they have evolved from a common ancestral receptor-ligand pair and that the conserved structural features of the repetitive cysteine-rich module underlie conserved common features of their action mechanisms (22–27).

Knowledge of the structural basis of TNF-R function is primarily confined to the p55-R species. X-ray analysis of co-crystals of its soluble form with TNF-β has provided information on the way in which p55-R binds TNF (28). Detailed mutational studies of its intracellular and "spacer" regions (the region that links its cysteine-rich module to the transmembrane domain) have revealed motifs that are involved in its signaling and in its induced shedding (29–32, and Brakebusch C., E. Varfolomeev, M. Batkin, and D. Wallach, manuscript in preparation). Many of the known effects of TNF can be induced in cells by the use of Abs against the extracellular domain of p55-R, indicating that the signaling activity of this receptor species suffices for their induction (e.g. 33, 34). The efficacy of induction is correlated with the extent of receptor cross-linking by the Abs, suggesting that initiation of the signaling activity of p55-R involves receptor aggregation (33).

The nature of the signaling mechanisms of p55-R and p75-R is not known. However, the lack of structural similarity between their intracellular domains suggests that they provide distinct signals. Indeed, several studies indicate that the two TNF-Rs can induce different effects (35–38). Other studies suggest, however, that at least some of the effects of TNF induced by p55-R, for example its cytocidal effect, are also affected by p75-R activity or activities (39–45). In some of these studies it was suggested that p75-R assists the induction of such TNF effects by enhancing the binding of TNF to p55-R (38), and in others that p75-R contributes via its own signaling activity (42, 44).

In this study, we explored the functional interactions of the two TNF-Rs by analyzing the effects of Abs against different regions in the extracellular domain of p75-R on TNF cytotoxicity. Our analysis confirmed that p75-R can participate in the cytocidal effect and indicated that its contribution includes two distinct kinds of activities. One of them, signaling for the cytocidal effect, known to be triggered by p55-R, was conclusively shown here to be triggered by p75-R as well. This signaling activity seems to emanate from the intracellular domain of p75-R and, like the signaling activity of p55-R, can be triggered by Ab-mediated receptor cross-linking. Second, by controlling the access of TNF to p55-R, p75-R appears to regulate p55-R–mediated cytotoxicity. This apparent activity of p75-R is also affected by Abs but is independent of receptor cross-linking. It appears to involve the extracellular domain of p75-R, not only in its membrane-distal part, to which TNF binds, but also in its membrane-proximal part, where a conformational change seems to take place upon TNF binding.

Materials and Methods

Abs and Their Characterization

A panel of 23 mAbs against human p75 TNF-R was established by immunizing mice with the soluble urinary receptor, as described for the development of Abs against p55-R (33). Cross-competition analysis of the binding of these Abs to the soluble receptor (33) showed that they bind to five epitopes, denoted here as epitope A (14 Abs), B (Abs number 31 and 41), C (36 and 62), D (67 and 81), and E (32, 57, and 70).

Monovalent Fab fragments of the mAbs were produced either by cleavage with papain, as described previously (33), or with pepsin, followed by reduction, alkylation, and purification on a protein G-Sepharose column (Pharmacia, Uppsala, Sweden) (46).

Rabbit polyclonal Abs against the spacer region in p75-R, which extends between the transmembrane domain and the COOH-terminal cysteine in the extracellular domain, were raised against a recombinant protein consisting of amino acids 181–235 of p75-R (numbered according to reference 47), fused to the maltose-binding protein (MBP). This fusion protein, produced using the pMal-cRI vector (New England Biolabs Inc., Beverly, MA), was expressed in protease-deficient BL21 bacteria and purified on an amylose column according to the manufacturer's instructions. The Abs were purified by ammonium sulfate precipitation and Abs against MBP were then removed by their adsorption on an irrelevant MBP fusion protein. Flow cytometry demonstrated their binding to the native receptor expressed by HeLa cells. Concentrations of these Abs are specified in terms of dilution, compared to their initial concentration in the serum.

Mouse monoclonal and rabbit polyclonal Abs against the soluble form of human p55-R were produced as described (2, 33). Rabbit polyclonal Abs against mouse p55-R were raised by immunizing rabbits with recombinant extracellular domain of mouse p55-R fused to MBP, produced using the pMal-p vector. The monoclonal anti-IL-6 Ab (number 34.1), used here as an isotype-matched negative control for the flow cytometry experiments, was a gift from Interlab Laboratories (Ness-Ziona, Israel). FITC-labeled goat Ab against the Fab fragment of mouse IgG was obtained from Biomakor (Rehovot, Israel). Goat Ab against mouse IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Affinity-purified goat anti-rabbit Ig, goat anti-rabbit Igs linked to horseradish peroxidase, and goat anti-mouse Igs linked to horseradish peroxidase were obtained from Biomakor.

Establishment of HeLa and A9 Cell Transfectants that Overexpress the Wild-type or Cytoplasmically Truncated p75-R

Human HeLa (48) and mouse A9 (49) cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cDNA for human p75-R was obtained by screening a λgt11 cDNA library of U937 cells (Clontech, Palo Alto, CA) with oligonucleotides corresponding to the published sequence of the receptor (7). The 5′ and 3′ untranslated regions were deleted (upstream from nucleotide 90 and downstream from nucleotide 1476, according to the numbering of reference 7), and the receptor was expressed in the pMPSVEH expression vector, under control of the myeloproliferative sarcoma virus promoter (50), either fully or after cytoplasmic deletion, downstream from Gln 264 or from Gln 273. The receptor constructs, together with the pSV2neo plasmid conferring resistance to neomycin, were transfected to the A9 and HeLa cells by the calcium phosphate precipitation method. Expression of the transfected receptor in clones resistant to G418 (500 μg/ml; Sigma Chemical Co., St. Louis, MO) was assessed by determining both the binding of radiolabeled TNF and that of monoclonal anti p75-R Abs to the cells. Levels of p75-R were quantified by measuring the binding of radiolabeled TNF at saturating concentrations to the cells in the presence of an Ab against p55-R (number 18). In all experiments, testing of cells of several clones overexpressing p75-R yielded qualitatively similar results.

The presented data concerning the HeLa cells that overexpress wild-type p75-R (HeLa p75 WT) refer to a specific clone that expressed about 72,000 TNF-R per cell. The effect of cytoplasmic truncation on p75-R function was tested in A9 cells by expressing the receptor truncated downstream from Gln 264. In HeLa cells, examination of mutants truncated downstream from Gln 264 and from Gln 273 revealed the same phenotype in both.

Assay of Cytocidal Activity of TNF and Abs against the Receptors

Recombinant human TNF-α (6×10^7 U/mg protein) was used, except in the indicated cases, where recombinant mouse TNF-α (4×10^7 U/mg protein) was employed. Both were produced by Genentech, Inc. (South San Francisco, CA) and kindly provided by Dr. G. Adolf (Boehringer Institute, Vienna, Austria). Unless otherwise stated, the cytocidal effects of TNF and of anti TNF-R Abs in the HeLa cells were assessed by applying TNF or the Abs to the cells for 10 h, in the presence of cycloheximide (CHI, 25 μg/ml). The A9 cells were treated with TNF or the Abs for 12 h, in the presence of 50 μg/ml CHI. Cell viability was determined by the neutral-red uptake method, as described elsewhere (51). The cytocidal effects of Abs against p75-R and of their Fab fragments after cross-linking with anti-Ig Abs, were assessed as described for Abs against p55-R (33). All tests were performed in triplicate. Viability of treated cells is presented as a percentage of the viable cells in cultures incubated with CHI alone ± standard deviation. Results of the cytotoxicity tests are representative of sets of at least three independent experiments that yielded similar results.

Quantification of TNF Binding to Cells and of its Modulation by Anti-p75 Abs

TNF was labeled with ^{125}I by the chloramine-T method, as previously described (52), to a specific radioactivity of 1,500 Ci/mmol. Specific binding of the radiolabeled TNF applied to the cells for 2 h at 4°C at a concentration of 0.5 nM, in the presence or absence of Abs against TNF-R, was determined as described (53).

The effects of anti p75-R Abs and of their Fab monovalent fragments on the rate of TNF dissociation from the receptors were assessed as follows: radiolabeled TNF was applied to the cells in PBS containing 154 mM NaCl, 10 mM sodium phosphate, pH 7.4, at a concentration of 1 nM, in the presence or absence of 10 μg/ml of the Abs. After incubation for 8 h, the cells were rinsed twice with ice-cold PBS to remove unbound TNF, and incubated further with PBS containing the Ab (10 μg/ml), 0.5% BSA, 0.02% sodium azide, and unlabeled TNF (0.1 μM), for the indicated time periods. They were then rinsed once with cold PBS, and the cell-bound radioactivity was determined. The data presented are average values for quadruplicate samples.

Quantification of Ab Binding to p75-R and of Its Modulation by Pretreatment with TNF

The extent and efficacy of binding of the various Abs to p75-R were assessed by the following three procedures:

Flow Cytometry. After treatment with TNF, nearly confluent HeLa p75WT cells were detached in PBS containing 5 mM EDTA and then incubated sequentially with anti p75-R Abs at various concentrations, and with FITC-labeled goat Ab against the Fab fragment of mouse IgG at a dilution of 1:20. Each incubation was carried out for 30 min at 4°C. The fluorescence intensity of the cells was analyzed using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Immunoprecipitation of the Receptor. Nearly confluent HeLa p75WT cells were treated with TNF and then rinsed with cold PBS containing 0.5% BSA. They were then incubated for 1.5 h on ice in the same buffer containing the indicated monoclonal anti p75-R Abs at a concentration of 10 μg/ml, or polyclonal Abs against p75-R spacer region at a dilution of 1:100. The cells were then washed, lysed, and extracted with RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA), supplemented with 1 mM PMSF and 10 mM benzamidine HCl. Insoluble material was pelleted by centrifugation for 30 min at 30,000 *g*. Ab–receptor complexes were adsorbed on protein G–Sepharose beads for 4 h at 4°C. The beads were washed thoroughly with RIPA buffer and PBS, resuspended, and boiled in SDS sample buffer containing mercaptoethanol. After SDS-PAGE (10% acrylamide), the proteins were Western blotted on nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) and probed with rabbit polyclonal Abs against the soluble form of p75-R, or (when immunoprecipitation was performed with antispacer Abs) with mAb number 32. The amount of Ab bound to the blots was determined by incubating the blots with goat anti-rabbit or goat anti-mouse Ab conjugated to horseradish peroxidase, and measuring the amount of bound conjugated Ab after further incubation with diaminobenzidine (Sigma Chemical Co.) and hydrogen peroxide.

Cell-ELISA of Abs Bound to the Cell in the Presence of Detergent. Confluent monolayers of HeLa p75WT cells in 96-microwell plates were treated with TNF, thoroughly rinsed with ice-cold PBS containing 1 mM calcium chloride and 1 mM magnesium chloride, and fixed with glutaraldehyde (0.1% in PBS) for 40 min at 4°C. Free aldehyde groups were blocked by incubation of the cells, first for 30 min at 37°C with 100 mM glycine in PBS, and then for 2 h with PBS, 0.5% BSA, 0.05% Tween-20, and 0.05% NaN_3. The plates were rinsed with PBS containing 0.05% Tween-20 and incubated in the same buffer for 2 h at 4°C, with the indicated concentrations of the Abs. The cells were rinsed with RIPA buffer, and the amounts of Ab bound to the cells were determined by incubating the cells with goat anti-mouse or goat anti-rabbit Ab conjugated to horseradish peroxidase followed by further incubation with 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co.) and hydrogen peroxide. Alternatively, Ab binding was quantified using preparations of Abs radiolabeled by the chloramine-T method (52). After binding of these Abs to the glutaraldehyde-fixed cell monolayers, the wells were rinsed with RIPA buffer, and Abs bound to the cells were detached in 2% SDS solution and quantified using a γ-counter.

Mapping of Epitope E. DNA sequences encoding different overlapping parts of the extracellular domain of p75-R were produced by PCR, using the full-length cDNA as template. The antisense primers used for the PCR reaction contained a stop codon. The DNA sequences were introduced into the pET8c vector (54) or were expressed in fusion with MBP by introduction into the pMalRI vector (New England Biolabs, Inc.). Bacteria (BL21) expressing these proteins upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG; Pharmacia) were resuspended in SDS sample buffer containing β-ME, denatured by boiling, and subjected to SDS-PAGE (10 or 12% acrylamide) followed by Western blotting. The blots were incubated sequentially with a blocking solution containing 154 mM NaCl, 10 mM sodium phosphate, pH 7.4, 0.05% Tween-20, 10% (vol/vol) bovine milk and 0.25% (vol/vol) normal goat serum, with an Ab against epitope E and with radiolabeled goat Ab to mouse IgG, and were then autoradiographed. Alternatively, binding of Abs to the blots was probed using goat anti-mouse Ab linked to horseradish peroxidase, as described above. The identity of the recombinant fragments of the

receptors was confirmed by determination of the molecular size of the IPTG-induced protein in the bacterial extracts by SDS-PAGE, and by interaction of the Western blot with polyclonal antiserum against soluble p75-R.

Involvement of the cysteine residues within epitope E in its recognition by Abs was explored in two ways: first, by determining whether the Abs interact with a recombinant protein corresponding to amino acids 3-181 of p75-R, where cysteine 179 was replaced by alanine. This protein (3-181 C → A) was produced and examined as described above, except that the antisense primer for PCR amplification contained the mutation (5' CTC GGA TCC TCA CGT GGA CGT [GGC] GAC TGC ATC CAT 3'). Second, the effect of alkylation of cysteine residues was assessed before and after reduction. A recombinant fusion protein, consisting of MBP fused to amino acids 125–192 of p75-R, was purified by adsorption on an amylose column and alkylated with iodoacetamide (75 mM for 1 h) before or after treatment with 100 mM dithiothreitol. It was then subjected to Western blotting as described above.

The mapping of the epitope was further confirmed by assessing the interaction of Abs against epitope E with synthetic peptides produced by t-Boc chemistry on a peptide synthesizer (model 430 A; Applied Biosystems, Foster City, CA). ELISA plates were coated with a fusion protein consisting of MBP fused to amino acids 125–192 in p75-R, produced as described above, and purified by adsorption on amylose. The protein, at a concentration of 1 μg/ml in PBS/0.02% sodium azide, was applied to the plates for 2 h. After further incubation of the plates for 2 h with a solution containing PBS, 0.5% BSA, and 0.02% sodium azide (blocking solution), an Ab against epitope E was applied for 2 h to the plates at a concentration of 1 μg/ml, with or without one of the synthetic peptides. The amounts of Ab bound to the protein-coated plate were determined by the use of goat anti-mouse Ig Ab conjugated to horseradish peroxidase and ABTS, as described above.

Results

Abs against p75-TNF-R Can Trigger Signaling for a Cytocidal Effect. HeLa cells predominantly express p55-R and are killed by agonistic Abs against this receptor species, but not by Abs against p75-R (Fig. 1 *A*). To examine the involvement of p75-R in the cytocidal function of TNF, we transfected HeLa cells with p75-R cDNA and isolated clones of cells expressing large amounts of the receptor (HeLa p75WT, Fig. 1 *C*). In these cells, Abs against p75-R triggered a mild cytocidal effect. Moreover, they potentiated the cytocidal activity of simultaneously applied Abs against p55-R (Fig. 1 *B*). The intensity of these effects varied among different transfected clones, roughly in proportion to their p75-R levels.

To investigate the mechanism whereby anti p75-R Abs exert their own cytocidal effect, we compared the ability of various individual Abs, or Ab combinations, to trigger such an effect. We employed 23 different mAbs raised against the soluble form of p75-R and antiserum raised against the spacer region in the receptor (a region not included in the soluble form of the receptor, extending between the cysteine-rich module in the extracellular domain and the transmembrane domain). Cross-competition analysis of their binding to soluble p75-R showed that the mAbs bind to five epitopes, denoted A to E. Table 1 presents a summary of the observed effects of the different Abs on HeLa p75WT cells, as well as on the other cells used in this study.

As shown in Fig. 2 *A*, Abs against p75-R were most effective when applied in combinations that bind to different epitopes. This increased efficacy was observed both when the anti p75-R Ab combinations were applied alone to HeLa p75WT cells and when applied in the presence of Abs against p55-R. Indeed, when applied alone, the anti p75-R Abs showed significant cytocidal activity only in such combinations. Abs against p55-R greatly potentiated the cytocidal effect of the anti p75-R Abs and in their presence the individual Abs and the combinations of Abs that bind to the same epitope also had some effect. The magnitude of the effect varied, depending on the epitope to which the Abs bound;

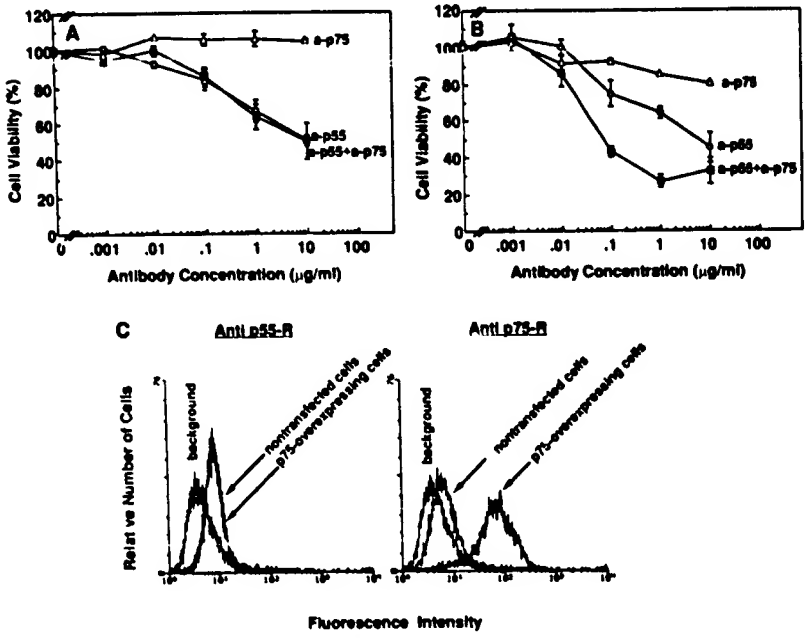

Figure 1. Additive cytocidal effects of p75-R and p55-R in HeLa cells transfected with p75-R. Comparison of the cytocidal effects of anti TNF-R Abs in (*A*) normal HeLa cells (expressing about 500 p75-Rs per cell) and (*B*) a clone of HeLa cells transfected with an expression vector containing p75-R cDNA (HeLa p75WT cells) and therefore expressing increased amounts of the receptor (about 72,000 p75-Rs per cell). The cytocidal effects of Abs against p55-R (○, mixture of Abs 18 and 20, which recognize distinct epitopes [33]), of Abs against p75-R (△, mixture of Abs 13, 36, and 41, which recognize distinct epitopes), and of their combination (■), applied at the indicated concentrations, were assessed as described in Materials and Methods. (*C*) Flow cytometric analysis of expression of p55 (*left*) and p75 (*right*) TNF-R in the normal and transfected HeLa cells examined in *A* and *B*. Cells were incubated consecutively with mAbs against p55-R (number 20) or p75-R (number 14), or with isotype-matched control anti-IL-6 Abs (*background*), all at a concentration of 20 μg/ml, and then with FITC-conjugated goat anti-mouse Ig Ab (see Materials and Methods for details).

Table 1. *Effects of Abs Against p75-R on HeLa and A9 Cell Expressing Wild-type and Cytoplasmically Truncated p75-R*

EPITOPE:	CYSTEINE-RICH MODULE A, B, C (18 Abs) Intact	CYSTEINE-RICH MODULE A, B, C (18 Abs) Fab	CYSTEINE-RICH MODULE D (2 Abs) Intact	CYSTEINE-RICH MODULE D (2 Abs) Fab	CYSTEINE-RICH MODULE E (2 Abs) Intact	CYSTEINE-RICH MODULE E (2 Abs) Fab	SPACER REGION Intact	SPACER REGION Fab
Ab EFFECTS INDICATING SIGNALING BY THE p75-R								
ON CYTOTOXICITY OF ANTI p75-R Abs								
IN HELA CELLS EXPRESSING WT p75-R	↑	—	↑	—	↑	—	↑	—
IN HELA CELLS EXPRESSING CT p75-R & IN A9 CELLS	—	—	—	—	—	—	—	—
INFERRED MODE F Ab ACTION	EFFECTIVE TRIGGERING		EFFECTIVE TRIGGERING		WEAK TRIGGERING		WEAK TRIGGERING	
Ab EFFECTS INDICATING CONTROL BY THE p75-R OF TNF AVAILABILITY TO THE p55-R								
ON CYTOTOXICITY OF TNF								
IN HELA CELLS EXPRESSING WT p75-R	↑	—	↑	↓	↓	↓	↓	↓
IN HELA CELLS EXPRESSING CT p75-R & IN A9 CELLS	↑	↑	↓	↓	↓	↓	↓	↓
Ab EFFECT ON TNF BINDING	↓	↓	↑	ND	↑	↑	↑	ND
TNF EFFECT ON Ab BINDING	—	—	↑	↑	↑	↑	↑	↑
INFERRED MODE F Ab ACTION	INCREASED AVAILABILITY OF TNF		DECREASED AVAILABILITY OF TNF		DECREASED AVAILABILITY OF TNF		DECREASED AVAILABILITY OF TNF	

↑, stimulation; ↑, weak stimulation; ↓, inhibition; —, No effect; ND, Not determined.

it was lowest, indeed barely detectable, with the Abs against epitope E and against the spacer region (Fig. 2, *A* and *C*).

Combinations of Abs that bind to different epitopes can cause extensive aggregation of the antigen molecules to which they bind, whereas Abs that bind to a single epitope are capable, at most, of linking the antigen molecules in couples. The greater cytocidal activity of combinations of Abs that bind to different epitopes in p75-R suggests that the receptors are triggered as a consequence of their cross-linking. This notion gained further support when we examined the cytocidal activity of Fab monovalent fragments of an Ab against epitope A. In contrast to the intact Ab, its Fab fragments were without cytocidal effect, even when applied in the presence of Abs against p55-R (Fig. 2 *B*). These Fab fragments did however have a cytocidal effect when cross-linked with anti-Ig Abs. Moreover, anti-Ig Abs enhanced the cytocidal activity of the intact Ab m lecules (Fig. 2 *B*). Also, cross-linking of the Abs against epitope E or the spacer region, which by themselves had almost no cytocidal effects, resulted in some cytotoxicity (Fig. 2 *C*).

Signaling by p75-R Appears to Interact with Signaling by p55-R. The more than additive effect of Abs against the two TNF-Rs suggests that, even though they initiate the same effect, they do so by different signaling mechanisms. To explore further the relationship between the mechanisms of cytotoxicity induction by the two receptors, we examined whether triggering of one of them can cross-desensitize the cells to the effect of the other. The cytocidal effect of Abs against p55-R was previously shown to be subject to homologous desensitization (33). As shown in Fig. 3, HeLa p75WT cells pretreated with anti p55-R Abs in the absence of a protein synthesis blocker (which in these cells is needed t elicit TNF cytotoxicity) showed almost no cytocidal effect following a second exposure to these Abs in the presence f a protein

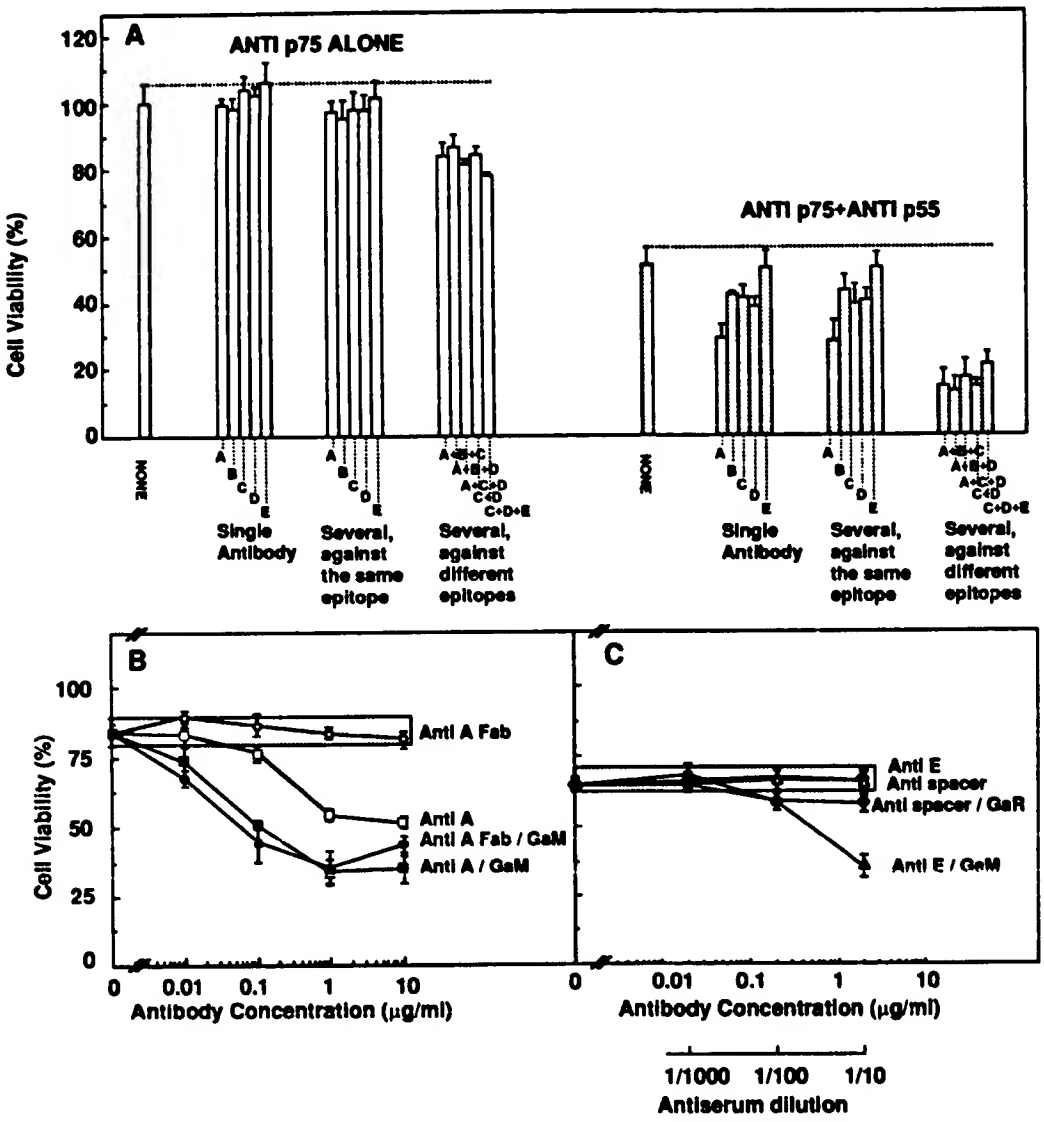

Figure 2. Cytocidal effects of Abs against p75-R in HeLa cells expressing wild-type p75-R reflect cross-linking of the receptors. (*A*) Cytocidal effects of individual Abs, combinations of Abs that bind to the same epitope, and combinations of Abs that bind to different epitopes in the absence (*left*) and presence (*right*) of Abs against p55-R. The cytocidal effects of the various anti-p75-R mAbs were assessed in HeLa p75WT cells, in the absence or presence of Abs against p55-R (numbers 18 and 20, both applied at a concentration of 1 μg/ml). Anti p75-R Abs were applied, each at a concentration of 2 μg/ml, individually (*Single Ab*: epitope *A*, number 14; epitope *B*, 41; epitope *C*, 36; epitope *D*, 67; epitope *E*, 32), in combinations of several Abs that bind to the same epitope (*Several against the same epitope*: epitope *A*, 14, 20, and 22; epitope *B*, 41, 47, and 82; epitope *C*, 36 and 62; epitope *D*, 67 and 81; and epitope *E*, 32, 57, and 70) or in combinations of Abs that bind to different epitopes (*Several against different epitopes*: *A* + *B* + *C*, 14, 41, and 36; *A* + *B* + *D*, 14, 41, and 67; *A* + *C* + *D*, 14, 36, and 67; *C* + *D*, 36 and 67; *C* + *D* + *E*, 36, 67, and 32). (*B*) Cytocidal effects on HeLa p75WT cells of an Ab against epitope A in p75-R (□, number 19) and of its Fab monovalent fragments (○), and their effects on these cells after their further cross-linking by goat anti-mouse Fab Abs (GaM, ●, ■; see Materials and Methods). The cytocidal effects were assessed in the presence of Abs against p55-R (rabbit polyclonal serum against the soluble form of the receptor (2), applied at a dilution of 1:500). (*C*) Cytocidal effects on HeLa p75WT cells of an Ab against epitope E in p75-R (△, number 32) and of antiserum against the spacer region in this receptor (◇), and effects on these cells after further cross-linking of the Abs with anti-Ig Abs (▲, ◆, goat anti-mouse Fab and goat anti-rabbit Ig Abs [*GaM* and *GaR*], respectively). The cytocidal effect of the mAb against epitope E was assessed in the presence of rabbit polyclonal Abs against p55-R (applied at a dilution of 1:250). The effect of the antiserum against the spacer region was determined in the presence of mouse mAbs against p55-R (numbers 18 and 20, both applied at concentration of 1 μg/ml). Dotted boxes in *B* and *C* indicate the range of the response observed when only the Abs against p55-R were applied.

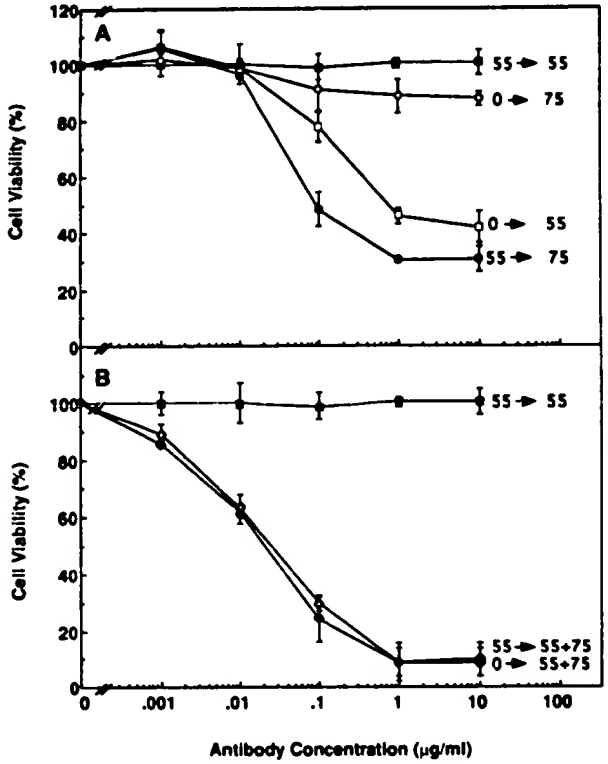

Figure 3. Absence of cross-desensitization by the two TNF-Rs to each other's cytocidal effects. HeLa p75WT cells were preincubated for 2 h, in the absence of CHI, with Abs against p55-R (numbers 18 and 20, each at a concentration of 1 μg/ml, *closed symbols*) or without anti-p55-R Abs (*open symbols*). The cytocidal effects of anti-p55-R Abs (□, ■, numbers 18 and 20), of anti p75-R Abs (○, ●, numbers 13, 36, and 41), and of their combination (◇, ◆), at the indicated concentrations, were subsequently assessed in the presence of 25 μg/ml CHI, as described in Materials and Methods.

synthesis blocker. They were not, however, desensitized to the effect of anti p75-R Abs. On the contrary, p75 treatment of cells desensitized to the effect of anti p55-R Abs resulted in a more than additive cytocidal effect, much greater than that induced in HeLa p75WT cells by Abs against either of the receptors alone (Fig. 3 *A*). A similar synergy was observed when cells pretreated with anti p75-R Abs were challenged with Abs against p55-R (data not shown). No desensitization was observed when cells pretreated with anti-p55 R Abs were challenged by Abs against both receptors (Fig. 3 *B*).

Effects of Anti-p75-R Abs on the Cytotoxicity of TNF Point to an Additional Mode of Receptor Function. Application of anti-p75-R Abs to HeLa p75WT cells in the presence of suboptimal TNF concentrations resulted, with most of the Abs, in enhancement of TNF cytotoxicity. This enhancement is consistent with the observed ability of the Abs to trigger a cytocidal effect when applied alone. As in that case, the enhancement appeared to be a consequence of receptor cross-linking by the Abs: it was most pronounced when the Abs were applied in combinations that bind to different epitopes (Fig. 4 *A*). Moreover, enhancement of TNF cytotoxicity was n t observed with Fab monovalent fragments of the Abs, but appeared when these fragments were cross-linked with anti-Ig (Fig. 4 *B*).

Unlike m st f the other Abs, however, the three that bind to epitope E did not enhance the cytotoxic activity of TNF, but rather inhibited it (Fig. 4, *A* and *C*). Also, the Abs raised against the spacer region had a slight though significant inhibitory effect on TNF function (Fig. 4 *C*). These inhibitory effects, unlike the enhancement of TNF cytotoxicity by the other Abs, were also observed when monovalent Fab fragments of the Abs were applied. This suggests the involvement of mechanism(s) independent of receptor cross-linking (Fig. 4 *C*). Some inhibition of the cytocidal effect of TNF was also observed in the presence of Fab monovalent fragments of the Abs against epitope D (Fig. 4 *C*), even though in the intact form these Abs enhanced TNF cytotoxicity (Fig. 4, *A* and *C*).

Whereas they inhibited the cytocidal effect induced by TNF, Abs against epitope E and against the spacer region did not interfere with the induction of a cytocidal effect by Abs against p55-R (Fig. 2 *C*), nor did they interfere with enhancement of the cytocidal effects of anti p55-R Abs or of TNF by other anti p75-R Abs. (Compare the effect of the combination of Abs that bind to epitopes C, D, and E with that of the combination of Abs against C and D only, in Figs. 2 *A* and 4 *A*.)

An Additional Mode of p75-R Function Is also Apparent in HeLa Cells Transfected with Cytoplasmically Truncated p75-R Mutants. To examine the involvement of the intracellular domain in the activities of p75-R, we transfected HeLa cells with cDNAs encoding cytoplasmically truncated mutants of the receptor (HeLa p75CT). In contrast to cells expressing large amounts of the wild-type p75-R (HeLa p75WT), which responded normally to the cytocidal effects of anti-p55-R Abs and of TNF, all clones expressing p75-R mutants (truncated below Gln 264 or Gln 273) were relatively resistant to such cytotoxicity. Anti-p75-R Abs had no effect at all when applied alone to cells expressing the receptor mutants, nor did they enhance the mild cytotoxicity of Abs against p55-R. Thus, as with p55-R (29–31), triggering of cytotoxicity by p75-R seems to depend on the integrity of the receptor's intracellular domain. A representative example of the data is presented in the legend to Fig. 4 *D*.

Although unable to initiate signaling for the cytocidal effect in HeLa p75CT cells, anti-p75-R Abs did affect the extent of cell killing by simultaneously applied TNF. Abs against epitope E or their Fab monovalent fragments inhibited the cytocidal effect of TNF, whereas Abs against epitope A enhanced it to a small but significant extent (Fig. 4 *D*). In contrast to the case where the Abs were applied together with TNF to HeLa p75WT cells, this latter enhancement could also be observed with Fab monovalent fragments of the Abs (Fig. 4 *D*); it therefore seems to involve not triggering of the signaling activity of p75-R, but some other mechanism(s), possibly related to the ability of the anti-A Abs to displace TNF from p75-R (see below).

Effects of Anti-p75-R Abs in A9 Cells Transfected with the Human Receptor Support a "Nonsignaling" Role of p75-R in TNF Function. To determine whether the effects f anti-p75-R

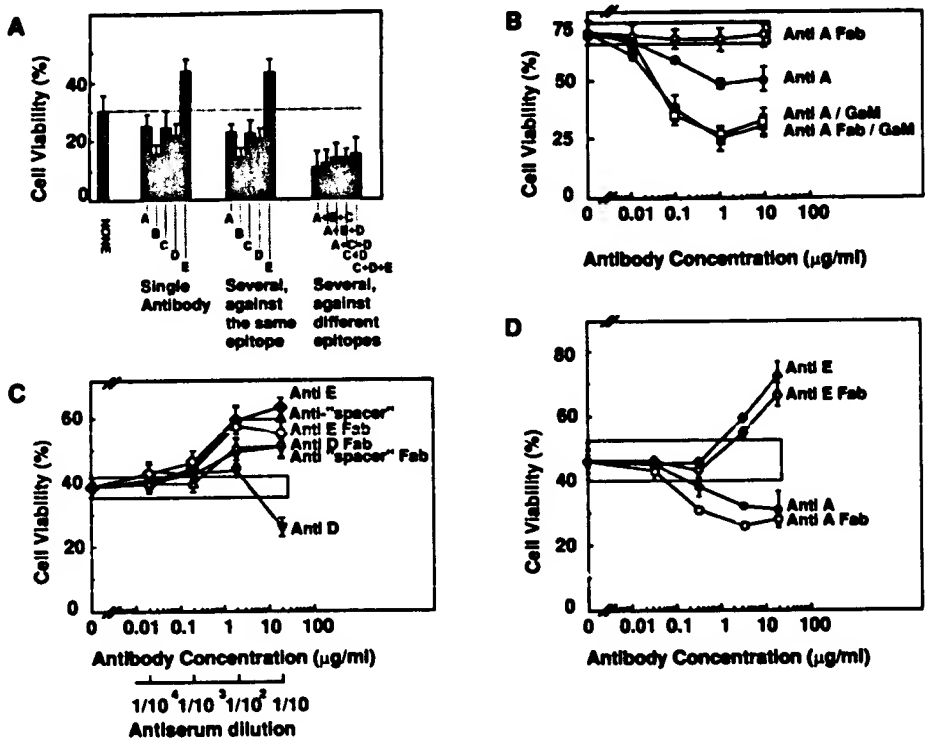

Figure 4. Enhancement and inhibition of the cytocidal effect of TNF by anti-p75-R Abs in HeLa cells expressing wild-type p75-R or a cytoplasmically truncated p75-R. (*A*) Effects on TNF cytotoxicity of individual Abs, combinations of Abs that bind to the same epitope, and combinations of Abs that bind to different epitopes. The cytocidal effects of the various monoclonal anti-p75-R Abs on HeLa p75WT cells were assessed in the presence of TNF (50 U/ml). The Abs and their concentrations are as in Fig. 2 *A*. (*B*) Effects of an Ab against epitope A (●, ■, number 19) and of its Fab monovalent fragments (○, □), with or without their cross-linking by goat anti-mouse Fab Abs (*GaM*), on the cytocidal effect of TNF (20 U/ml) in HeLa p75WT cells. (*C*) Effects of Abs against epitope D (▼, number 67) and E (◆, number 32), antiserum against the spacer region in p75-R (▲) and Fab monovalent fragments of these Abs (▽, ◇, △) on the cytocidal effect of TNF (50 U/ml) in HeLa p75WT cells. (*D*) Effects of an Ab against epitope A (●, number 13) and of its Fab monovalent fragments (○) and effects of an Ab against epitope E (◆, number 32) and of its Fab monovalent fragments (◇) on the cytocidal effect of TNF (1,000 U/ml) in HeLa cells expressing cytoplasmically truncated p75-R (HeLa p75CT, about 140,000 receptors per cell). When applied alone to the HeLa p75CT cells, anti-p75-R Abs (numbers 14, 62, and 82, each at a concentration of 20 μg/ml) had no effect on TNF cytotoxicity, nor did they enhance the cytotoxicity induced by anti-p55-R Abs (numbers 18 and 20, each applied at a concentration of 20 μg/ml, which caused killing of 17 ± 3% of the cells in this experiment). Dotted boxes in *B–D* indicate the range of the response observed when TNF was applied alone to the cells.

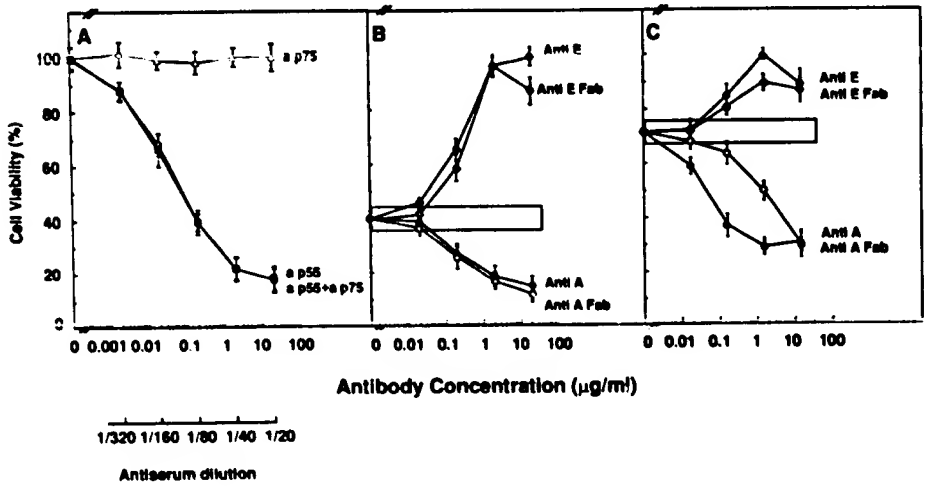

Figure 5. Anti-p75-R Abs have no cytocidal effect in A9 cells expressing high levels of p75-R but do modulate the cytocidal effect of TNF on these cells. (*A*) Lack of a cytocidal effect of anti-p75-R Abs (△, mixture of Abs 13, 36, and 41) on A9 cells expressing large amounts of full-length p75-R (A9 p75WT, about 90,000 receptors per cell), in contrast to the marked cytotoxicity of an antiserum against the murine p55-R (○). Anti-p75-R Abs also fail to enhance the cytotoxicity of the anti murine p55-R antiserum (■). (*B* and *C*) Enhancement of the cytocidal effect of TNF (1,000 U/ml) by an Ab against epitope A (●, number 13) and by its Fab monovalent fragments (○), and inhibition of the effect by an Ab against epitope E (◆, number 32) and by its Fab monovalent fragments (◇) in A9 cells expressing wild-type or cytoplasmically truncated human p75-R. (*B*) A test performed with the same A9 p75WT clone that was tested in *A*. (*C*) A test performed with A9 p75CT (~110,000 receptors per cell). Abs against epitope B and, to a lesser extent, Abs against epitope C also enhanced the cytocidal effect of TNF in A9 p75WT or A9 p75CT cells, whereas Abs against epitope D and against the spacer region had inhibitory effects similar to those of the Abs against epitope E. The Abs had the same effects when mouse rather than human TNF was used. Dotted boxes in *B* and *C* indicate the range of the response observed when TNF was applied alone to the cells.

Abs bserved with HeLa cells also pertain to other cells, we examined their effect n mouse A9 cells transfected with cDNA for the wild-type human p75-R (A9 p75WT) or for a cytoplasmically truncated mutant f it (A9 p75CT). All transfected clones exhibited a significantly reduced cytotoxic response to TNF, in correlation with the amounts of the expressed receptor, but responded normally to the cytocidal effect f Abs against their murine p55-R. Unlike the HeLa p75WT cells, A9 p75WT and A9 p75CT cells did not exhibit a cytocidal effect when treated with Abs against human p75-R, nor did these Abs enhance the cytocidal effect induced in the cells by Abs against murine p55-R (Fig. 5 *A* and data not shown).

Although unable to initiate signaling for a cytocidal effect in A9 p75WT or A9 p75CT cells, anti-p75-R Abs strongly affected the killing of these cells by TNF. These effects resembled those observed in HeLa p75CT cells. Abs or Fab monovalent fragments that bind to epitopes D or E, or to the spacer region, reduced the cytotoxic response of the cells to TNF. Abs or Fab fragments that bind to epitopes A and B and (though to a lesser extent) Abs or Fab fragments that bind to epitopes C enhanced the cytocidal effect of TNF in these cells (Fig. 5, *B* and *C* and data not shown).

Effects of Anti-p75-R Abs on TNF Binding to p75-R. Abs binding to different epitopes in p75-R affected TNF binding in quite different ways. The pattern of these effects was almost opposite to that of their effects on TNF function (Fig. 6). Abs that bind to epitopes A, B, or C, which enhanced TNF function in HeLa p75WT cells, were found to inhibit TNF binding. This inhibitory effect was greater with the Abs that bind to epitopes A or B than to C. However, Abs against epitopes D or E or the spacer region, all of which inhibited TNF function, did not inhibit its binding. In fact, cells exposed to Abs against epitope E exhibited some increase in TNF binding (Fig. 6 *A*). Moreover, measurement of the dissociation of TNF from p75-R, which occurs rather rapidly (38), revealed that Abs against epitopes D or E and, to a lesser extent, Abs against the spacer region inhibit the dissociation of TNF from the receptor. Fab monovalent fragments of the Abs against epitope E had a similar inhibitory effect (Fig. 6 *B*).

Effects of TNF on Binding of Anti-p75-R Abs to Epitopes D and E and to the Spacer Region. As shown above, Abs against epitopes D or E or the spacer region affected TNF binding and function even when in their monovalent form. This suggests that their effects are related to the conformation of the receptor molecules rather than to their extent of aggregation. The fact that these Abs affect TNF binding and function even though the site at which they bind to p75-R seems remote from the one at which TNF binding takes place suggested that the conformation of the receptor at the Ab binding site is affected by TNF. To test these notions, we compared the ability of different Abs against the receptor to bind to it before and after TNF binding. Three kinds of tests were employed (see Materials and Methods). In the first, the actual binding of the Abs to HeLa p75WT cells was quantified by flow cytometry. No difference between TNF-treated and

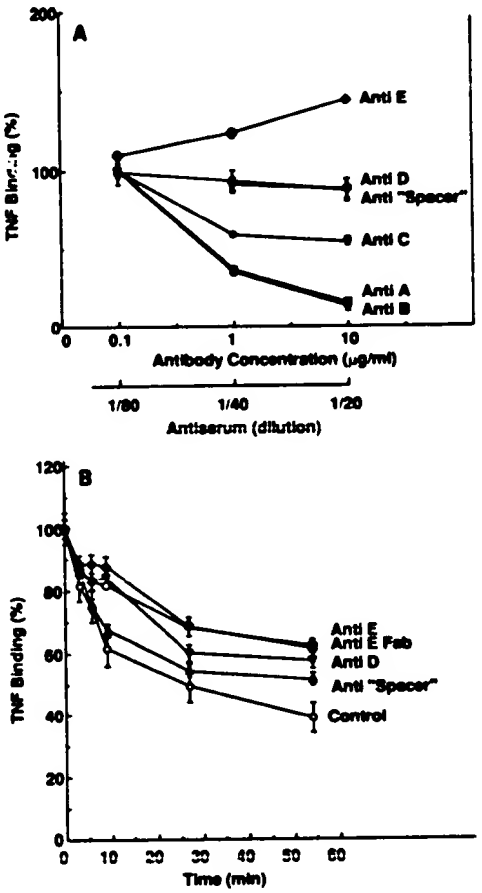

Figure 6. Inhibition or enhancement of TNF binding to p75-R by the various anti-p75-R Abs. (*A*) Effects of the various anti-p75-R Abs on TNF binding. The effects of Abs against epitope A (■, number 14), B (□, −41), C (●, −36) D (▼, −67) and E (◆, −32) and of Abs against the spacer region (▲) on the binding of radiolabeled TNF to HeLa p75WT cells were assessed as described in Materials and Methods. All Abs binding to the same epitope affected TNF binding similarly (data not shown). (*B*) Kinetics of the dissociation of radiolabeled TNF from HeLa p75WT cells in the absence of Abs (○) and in the presence of an Ab against epitope D (▼, number 67) or E (◆, −70) or its Fab monovalent fragments (◇, all applied at a concentration of 10 μg/ml) or of Abs against the spacer region (▲, at a dilution of 1:20).

untreated cells was observed (data not shown). The other two tests probed the efficacy of Ab binding to p75-R by subjecting the Ab receptor complexes to detergent treatment. In one, binding efficacy was evaluated by assessing the recovery of the receptor immunoprecipitated from detergent extracts of HeLa p75WT cells. In the other, the amounts of Abs bound to the receptor were quantified by a cell-ELISA procedure.

In the latter two tests, TNF was found to enhance the binding to the receptor of Abs against epitopes E or D or the spacer region, but not that of Abs against the other epitopes. TNF treatment increased the efficacy with which these Abs and their Fab m novalent fragments could immun precipitate p75-R from detergent extracts f the cells. No such

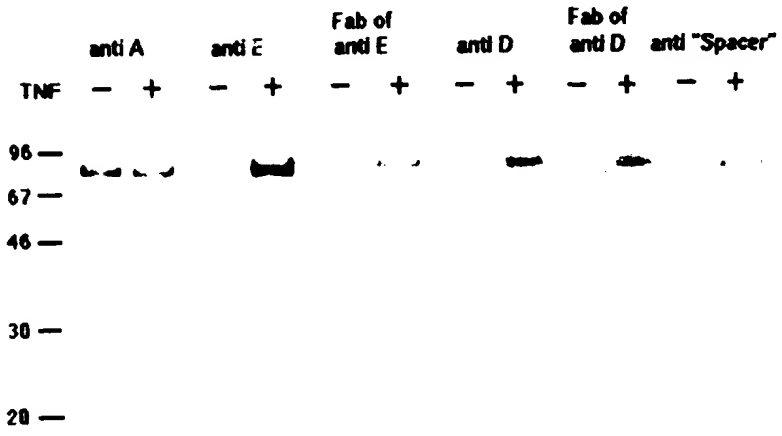

Figure 7. TNF enhances the binding of Abs to epitopes D and E and to the spacer region of p75-R: assessment by immunoprecipitation. Subconfluent cultures of HeLa p75WT cells were treated with TNF (10,000 U/ml) for 10 min. They were then incubated for 90 min at 4°C with the following Abs: an Ab against epitope A (number 13), an Ab against epitope E (number 32) or its Fab monovalent fragments, an Ab against epitope D (number 67) or its Fab monovalent fragments, each at a concentration of 10 μg/ml, or Abs against the spacer region at a dilution of 1:100. Unbound Abs were washed off and the cells were lysed with RIPA buffer, followed by precipitation of the receptor–Ab complexes, SDS-PAGE (10%) and Western blotting, as described in Materials and Methods.

effect was observed with Abs against epitopes A, B, or C (Fig. 7 and data not shown). TNF treatment also significantly increased the efficacy with which Abs against epitopes E or D or against the spacer region, but not Abs against A, B, or C, bound to (glutaraldehyde-fixed) cells in a cell-ELISA test where cells were rinsed with a detergent solution (Fig. 8 and data not shown). These effects were specific for TNF. They were observed with both TNF-α and TNF-β (lymphotoxin), but not after treatment with IL-1α, which in many respects functions similarly to TNF, or with IFN-γ, shown to enhance TNF-R expression in HeLa cells (52). In a kinetic study, they could be discerned as early as 1 min after TNF was applied to the cells and reached a maximum within 5 min (data not shown).

Mapping of Epitope E. The three Abs against epitope E (but none of the 20 other mAbs against p75-R) recognized p75-R in Western blot analysis, even when the receptor molecules were analyzed after their complete denaturation and reduction. This enabled us to map epitope E by Western blot analysis of the binding of the Abs to various bacterially expressed deletion mutants of the extracellular domain of p75-R (Fig. 9 *B*). We found (Fig. 9 *A*) that NH_2-terminal truncation of the receptor to Cys 163 and COOH-terminal truncation to Thr 179 did not affect recognition by the three Abs. However, all further truncations prevented recognition, suggesting that epitope E corresponds to the part of the protein that extends between the residues Cys 163 and Thr 179. Interaction of the Abs with a synthetic peptide consisting of amino acids 163-179 confirmed this identification (Fig. 9 *C*).

As with reduction and denaturation, alkylation with iodoacetamide did not affect recognition of the receptor by Abs against epitope E in Western blot analysis. However, recognition was completely prevented when the receptor was first reduced and then immediately alkylated (see Materials and Methods; data not shown). Recognition was also prevented by mutational replacement of one of the cysteine residues in the epitope region by alanine (3-181 C → A in Fig. 9 *B*). These findings indicated that the structure to which the Abs bind is not the reduced form of the protein, but the cystine loop formed between the cysteine residues at positions 163 and 178. The fact that this protein can be recognized in Western blotting after its complete reduction and denaturation suggests that the primary structure of the protein in this region dictates correct refolding of the denatured receptor, as well as reformation of the cysteine link.

The mapping of epitope E to the COOH-terminal end of the cysteine-rich module points to a correlation between the effects of the various Abs against the extracellular domain of p75-R on TNF function and the location of the epitope to which they bind (Table 1). Abs that bind to the membrane-proximal part of the extracellular domain—to its COOH-terminal cysteine loop and the spacer region—have different effects on TNF function and binding from those of most of the Abs that bind to the membrane-distal part. Only the two Abs that bind to epitope D showed a mixed type of effect, raising the possibility that this epitope is located at the boundary between the two regions.

Discussion

Involvement of p75-R in the cytocidal effect of TNF was explored in this study by probing the function of this receptor with Abs that bind to different regions in its extracellular domain. The results (summarized in Table 1) indicated that p75-R contributes to the cytocidal effect by at least two kinds of activities: its own signaling activity and control of the access of TNF to p55-R.

Examination of the effects of Abs on the viability of HeLa cells manipulated to express large amounts of wild-type p75-R indicated that p75-R plays a role in signaling for the cytocidal effect of TNF. Anti-p75-R Abs were cytotoxic to these cells even in the absence of TNF or anti-p55-R Abs, and enhanced the cytocidal effect of anti-p55-R Abs. These effects of the anti-p75-R Abs were correlated with their ability t cross-link the receptor molecules, suggesting that the signaling occurs as a consequence f receptor aggregation. T some extent, these effects were als dependent on the site to which the Abs bind within the recept r. Abs against epi-

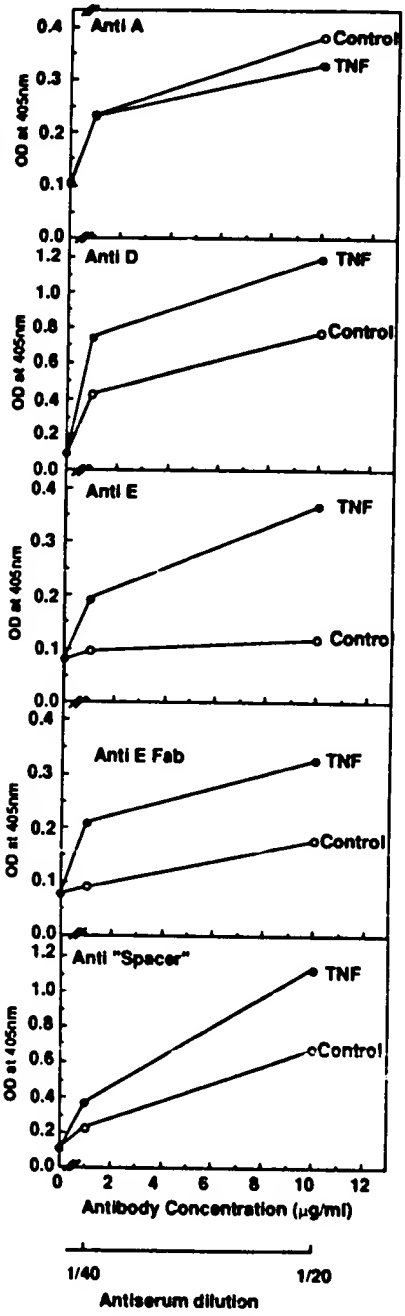

Figure 8. TNF enhances the binding of Abs to epitopes D or E and to the spacer region of p75-R: assessment by cell-ELISA. Effects of TNF on the binding of mAbs against epitopes A (number 13), D (number 67), and E (number 32), of Fab monovalent fragments of the Ab against epitope E, and of Abs against the spacer region. TNF (10,000 U/ml) was applied to HeLa p75WT cells for 10 min. The cells were then fixed with glutaraldehyde. Binding of the Abs applied at the indicated concentrations, after washing of unbound Abs with RIPA buffer, was assessed as described in Materials and Methods. Similar results were obtained when the binding of Ab number 32 or of its Fab fragments was quantified by applying radiolabeled preparations of these Abs to the cells.

topes A, B, C, and D, at the membrane-distal part f the receptor's extracellular domain, had stronger effects than those that bind t epitope E or to the spacer region (Fig. 2). However, the epitope E and anti-spacer Abs showed increased cytotoxicity when their ability to cross-link the receptor molecules was enhanced by their cross-linking with anti-Ig Abs. The partial site dependence of their effects may reflect differences in the ability of the Abs to impose an orientation n the receptor molecules resembling that attained when TNF binds to them. Abs that bind to epitopes A, B, C, and D may be better able to bring about this orientation, possibly because their binding sites to the receptor are close to the region at which TNF binds (as reflected in the ability of the Abs that bind to epitopes A, B, and C to interfere with TNF binding, Fig. 6 *A*).

Similar to the signaling activity of p75-R, signaling for the cytocidal effect by p55-R is triggered upon receptor aggregation (33). Yet, in spite of this similarity in mode of triggering of the receptors, and the apparent identity of their impact on the cell, it seems that the mechanisms of signaling for cell death by the two receptors differ, at least at their initial step. This difference was evident from the way in which stimulation of each receptor affected the activity of the other. HeLa cells prestimulated with Abs against p55-R became almost completely unresponsive to the cytocidal effect of these Abs (33, and the present study), yet showed a response greater than the additive effect of the two receptors when subsequently stimulated with Abs against p75-R. It thus seems that the signals provided by p75-R are able to reverse the desensitization of p55-R or perhaps to uncover preformed p55-R–mediated signals in the desensitized cells and act in concert with them. The possible occurrence of receptor-specific desensitization is consistent with a prior study which indicated that desensitization of cells to TNF cytotoxicity (51, 55, 56) involves, in addition to activities that antagonize the cytocidal effect itself (57–59), activities that inhibit signaling-related mechanisms (60).

Although capable of triggering a cytocidal effect in HeLa cells, the anti-p75-R Abs were unable to do so in A9 cells, irrespective of whether the transfected human p75-R that they expressed was of full-length or cytoplasmically truncated. The nature of this difference between the A9 and the HeLa cells is not clear. It is unlikely to reflect a species specificity barrier, as human p75-R does have the ability to trigger TNF effects in mouse cells (37). Moreover, a recent study (38) demonstrated that Abs against endogenous mouse p75-R cannot trigger a cytocidal effect in cells of the mouse L929 line, which are closely related to the A9 cells. Perhaps the difference is related to the way in which TNF cytotoxicity is regulated in the two cell lines. In L929 and A9 cells, unlike in HeLa cells, preexposure to TNF or to Abs against p55-R does not result in desensitization (55, and data not shown). It thus appears that the desensitization mechanisms which restrict the signaling for cell death in HeLa cells, and which seem t be reversed upon triggering of p75-R, do not operate in A9 cells.

Effects f the anti p75-R Abs n TNF ct toxicity were correlated nly in part with their own ability to trigger cyto-

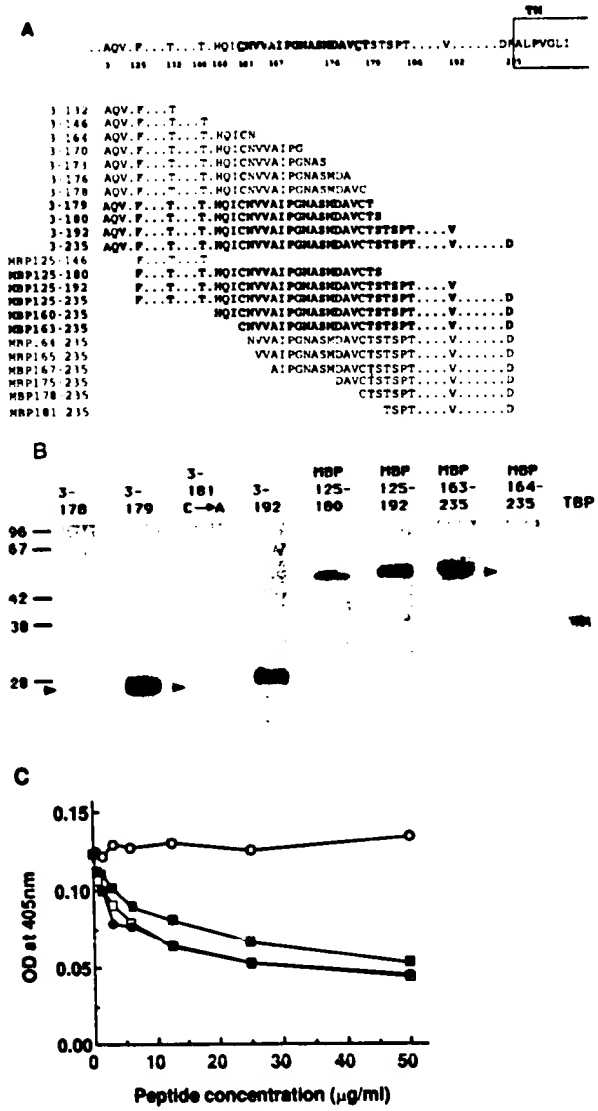

Figure 9. Mapping of epitope E. (*A*) Schematic representation of the bacterial constructs used for mapping. Recombinant peptides corresponding to parts of the extracellular domain of p75-R were expressed in *Escherichia coli*. Constructs differing in their COOH termini were obtained by use of the pET8c vector, whereas products differing in their NH_2-termini were obtained as fusion proteins with the maltose binding protein (*MBP* constructs) by use of the pMalcRI vector. Recognition of these contructs by Abs against epitope E was assessed by Western blot analysis, as described in Materials and Methods and exemplified in *B* below. Constructs that reacted with the Abs are denoted in bold letters. The three mAbs against epitope E exhibited identical reaction patterns with the constructs. The sequence of amino acid residues in the extracellular domain of p75-R is shown in part. Residues found to be within the region of epitope E are denoted in bold letters. The two cysteine residues in it are underlined. (*TM*) Transmembrane domain. (*B*) An example of the Western blot used to map epitope E. After IPTG induction, samples (0.1 ml) of bacterial cultures expressing the indicated products were lysed in SDS-PAGE sample buffer and analyzed by SDS-PAGE (12% acrylamide). Western blotting was performed with mAb number 32 and goat anti-mouse Igs linked to horseradish peroxidase in the first and second steps of probing, respectively, as described in Materials and Methods. The urine-derived soluble form of p75-R (*TBP*, 0.36 μg [2]) served as a positive control. (*Arrowheads*) Location of the recombinant peptides that did not react with the Ab. This location was defined both by Coomassie blue staining and by Western blot analysis of the proteins, using rabbit polyclonal Abs against the soluble form of p75-R (2). (*Left*) Molecular weight markers. (*C*) ELISA for the binding of synthetic peptides by an Ab against epitope E. Comparison for binding to Ab number 32 between peptides whose sequences correspond to residues 165-180 (○), 163-180 (■), 162-180 (□), or 160-180 (●) in p75-R and a recombinant protein that contains the sequence of epitope E (a fusion protein comprised of MBP linked to a recombinant protein corresponding to amino acids 125-192 in p75-R) was assessed as described in Materials and Methods. Similar results were obtained with the two other Abs against epitope E.

cidal activity, al ne or in the presence of anti-p55-R Abs (see Table 1). The partial nature f these correlati ns suggests that p75-R contributes t the cytocidal functi n of TNF not nly by its signaling activity, but also in an ther manner. The differences between the signaling effects of the Abs al ne and their overall effects on TNF cytotoxicity were particularly notable in the transfected A9 cells and in HeLa cells expressing cytoplasmically truncated p75-R. Even though in these cells the Abs themselves did not trigger signaling, they had a significant effect on the killing of the cells by TNF. This was true also when the Abs were applied as Fab monovalent fragments (Table 1), indicating that this Ab-induced modulation, unlike triggering of the signaling activity of p75-R, does not involve cross-linking of p75-R. Two ways of modulation, depending on the site of Ab binding to the receptor, were observed. Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, i.e., to its COOH-terminal cysteine loop or to the spacer region, inhibited the cytocidal effect of TNF. This inhibition was observed in all transfectants studied, including the HeLa p75WT cells. Abs that bind to epitopes A, B, or C at the membrane-distal part of the extracellular domain, and which in HeLa p75WT cells triggered signaling for cytotoxicity, enhanced the cytocidal effect also in cells that could not respond to signaling by p75-R (A9 cells and HeLa p75CT). In the latter cells, all the above Ab effects on TNF cytotoxicity were also observed when the Abs were applied as Fab monovalent fragments (Table 1).

Several points of evidence indicate that these two ways of modulation reflect an ability of p75-R to control the access of TNF to p55-R. (*a*) Because modulation was also observated in cells in which p75-R could not signal for cytotoxicity, it must reflect the effect(s) of p75-R (specifically, of its extracellular domain) on the cytotoxicity signaled by p55-R. (*b*) The modulation affected only the triggering, not the actual mechanism of signaling. Cells whose killing was triggered by agonistic Abs against p55-R showed no protection from the killing when treated with Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, nor enhancement of it when treated with Fab monovalent fragments of Abs that bind to the distal part of the extracellular domain. (*c*) Modulation of TNF function by the different Abs was inversely related to their effect on TNF binding to p75-R. Potentiation of TNF activity was observed with those Abs that displace TNF from p75-R, and inhibition of TNF function was seen with Abs that enhance its binding.

Two possible mechanisms by which the access of a ligand

t its recept r can be controlled by ther, coexpressed, receptors were recently proposed. One was presented in a study that, like the present ne, concerned the interacti ns of the two TNF-Rs (38), and the other in a study of the interactions of the two receptors for IL-1 (61). The two proposed mechanisms have practically opposing consequences. According to the first, interactions between the two receptors can result in enhancement of signaling; it was suggested that the p75 TNF-R is capable of "ligand passing", i.e., of presenting TNF to p55-R in such a way that its binding to p55-R is enhanced. The second mechanism can account for inhibitory interactions between two receptors. It was suggested that the type II IL-1 receptor serves as a "decoy receptor" which inhibits the binding of IL-1 to the type 1 IL-1 receptor by its own ability to bind IL-1. Whether and to what extent either of these two ways of modulation contributes to the observations of the present study is not clear. Perhaps both are involved.

The enhancement of TNF cytotoxicity by its displacement from p75-R with Abs against epitopes A, B, and C (in those transfectants where p75-R cannot signal) indicates that this receptor can have an inhibitory effect on the function of p55-R. A "ligand passing" mechanism cannot account for this effect since it should result not in inhibition but in enhancement of signaling. The inhibitory effect can however be explained by assuming that p75-R functions as a decoy receptor. At the levels attained in the transfected cells of this study, this receptor, by binding TNF, may have decreased the concentration of TNF in the cell culture and thus reduced the activation of p55-R.

On the other hand, the inhibition of TNF cytotoxicity by Abs that bind to the membrane-proximal part of the extracellular domain of p75-R is unlikely to be due to its function as a decoy receptor. Although these Abs decrease the dissociation of TNF from p75-R, this decrease is rather mild and results in only a small enhancement of TNF binding to the cells (Fig. 6 *A*), without any significant decrease in the concentration of TNF in the cell growth media (our unpublished data). Perhaps the Abs, by inhibiting dissociation of TNF from p75-R, slow down ligand passing between the two receptors (38).

Even though the action of decoy receptors and the process of ligand passing result in opposing effects, they may well co-occur, since they relate to different ways of ligand binding. Decoy receptors affect the access of ligands in the cell's milieu. The way in which ligand passing occurs is not clear, but it seems plausible that it occurs by transient formation of a ternary complex of the ligand with the two receptors, without involvement of the ligand found in the fluid phase compartment.

The way in which binding of Abs to the membrane-proximal part of p75-R, quite remote from the ligand-binding site, decreases the rate of TNF dissociation from the receptor remains to be clarified. The data presented in this paper indicate that these Abs and TNF have mutually positive effects in their binding to the receptor. The am unts of the Abs that bound to the membrane-proximal part of the extracellular d main were the same in cells treated or untreated with TNF. However, the increased ability f the Abs to remain associated with the TNF-bound receptor in the presence of detergent indicates an increased efficacy of Ab binding. A likely explanation for the mutual effects of the Abs and TNF in their interaction with the receptor is that the binding of Abs to the membrane-proximal part of the extracellular domain and the binding of TNF to the distal part cause similar conformational changes in the receptor, and thus enhance each other allosterically. Induced conformational changes have also been observed in the extracellular domains of a number of other receptors as a consequence of their binding to their respective agonists (62–64). Notably, both the structure of epitope E (cysteine loop) and its size (17 residues, significantly above the size characteristic of sequence epitopes) would allow it sensitively to reflect changes in receptor conformation.

The findings of this study, although obtained only by examining the cytocidal activity of TNF, have general implications for our understanding of the function of the two TNF-Rs. The ability of p75-R to participate doubly in an effect signaled by p55-R—both by controlling the access of TNF to p55-R and by its own signaling activity—implies that, even if the two TNF-Rs can be triggered separately, they constitute part of one functional unit. The ways in which the expression and function of p75-R are regulated are therefore likely to affect not only those TNF activities that are triggered by this particular receptor, but also the intensity of the effects of TNF signaled by p55-R. Moreover, pharmaceutical agents affecting p75-R may have a general impact on TNF function, including those activities whose major signaling receptor is p55-R. Such pharmacological modulation is perhaps indicated by the mode of function of Abs that bind to the membrane-proximal part of the extracellular domain of p75-R, particularly those that bind to its COOH-terminal cysteine loop. Because they are able to inhibit the activation of p55-R by TNF, and are unable to trigger the signaling activity of p75-R (see Fig. 2), these Abs may be useful as inhibitors of TNF function. Particularly suitable targets for such inhibition are cells of the monocytic and lymphocytic lineages, where the predominant receptor is p75-R yet many of the TNF effects depend on the signaling activity of p55-R (65). Preliminary findings in our laboratory indicate that Abs against epitope E can inhibit the effects of TNF in such cells, just as in the transfected cells examined in the present study, even though TNF binding to them is enhanced. In view of the marked conservation of various structural features in the cysteine-rich module that characterizes the NGF/TNF receptor family, particularly the location of the cysteine residues, it seems plausible that these structural features have similar functions in the different members of this family. It would be of interest to determine whether the COOH-terminal cysteine loops in the extracellular domains of the various other receptors of this family display features similar t those observed in p75-R and are affected in the same way by Ab binding.

We thank Adela Dibeman, Ala Feldmann, and Serge Lindner for their assistance in the isolation and maintenance of the transfected cells, and Shlomit Maizel for her help in the production and purification of Abs. We are grateful to Oliver Kemper and Dr. Talia Sher for critical reading of the manuscript.

This work was supported in part by grants from Inter-Lab Ltd., Ness-Ziona, Israel and from Ares Trading S.A., Switzerland. J. Bigda is a recipient of a fellowship from the Feinberg Graduate School, The Weizmann Institute of Science. Yevgeny Varfolomeev is a recipient of the International Cancer Technology Transfer award from the International Union Against Cancer, Geneva, Switzerland.

Address correspondence to Dr. D. Wallach, Department of Membrane Research and Biophysics, The Weizmann Institute of Science, 76100 Rehovot, Israel. J. Bigda's permanent address is Department of Histology, Gdansk School of Medicine, 80-210 Gdansk, Poland. I. Beletsky is currently at LXR Biotechnology Inc., Richmond, CA 94804; C. Brakebusch is currently at Department of Molecular Biology, Max-Planck Institute, 8033 Martinsried bei München, Germany; and H. Engelmann is currently at Institute of Immunology, University of Munich, 8000 Munich 2, Germany.

Received for publication 10 January 1994 and in revised form 18 April 1994.

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Journal code: A52. ISSN: 1043-4666.

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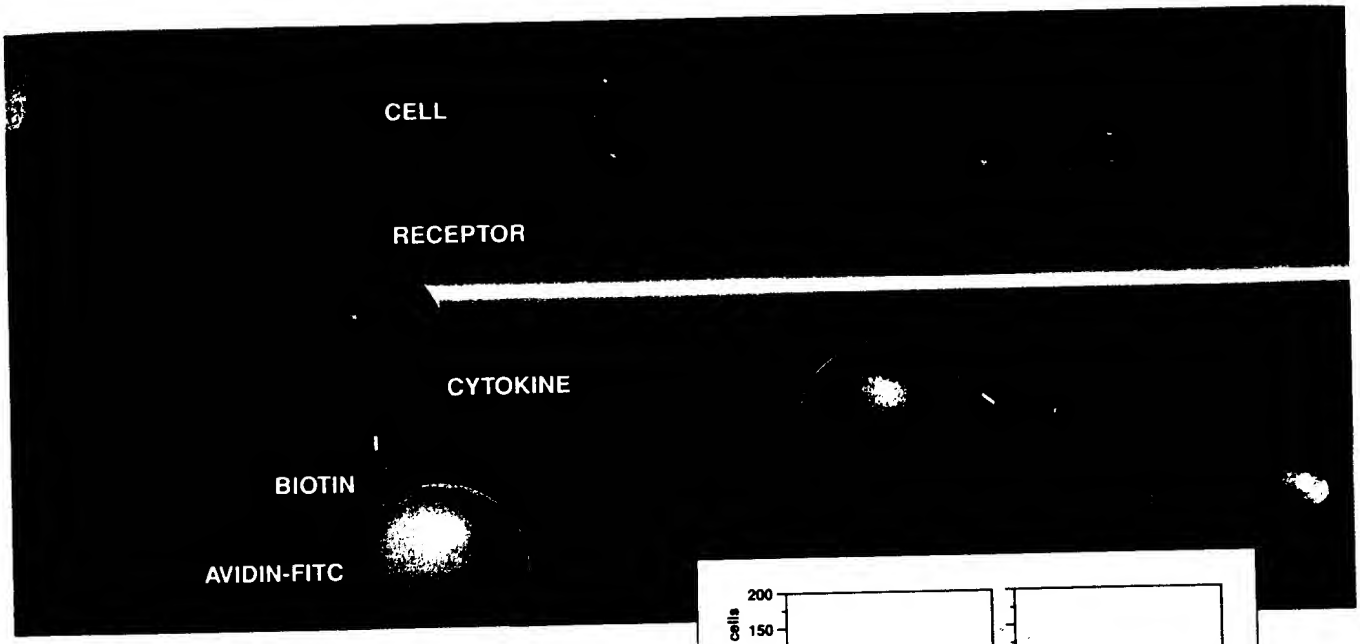

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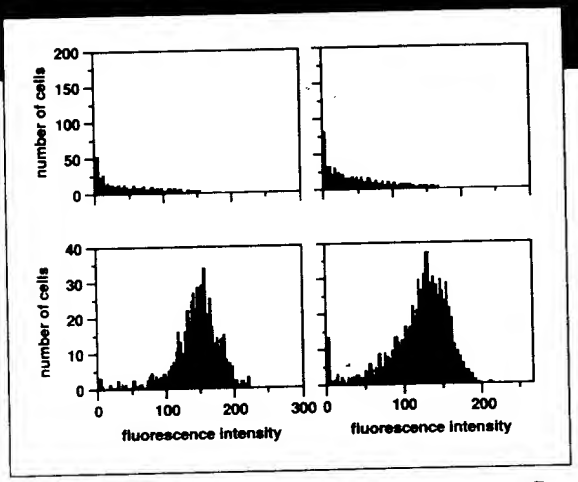

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TWO HUMAN TNF RECEPTORS HAVE SIMILAR EXTRACELLULAR, BUT DISTINCT INTRACELLULAR, DOMAIN SEQUENCES

Zlatko Dembic,[1] Hansruedi Loetscher,[1] Ueli Gubler,[2] Yu-Ching E. Pan,[2] Hans-Werner Lahm,[1] Reiner Gentz,[1] Manfred Brockhaus,[1] Werner Lesslauer[1,*]

Tumor necrosis factor (TNF) is a cytokine with a wide range of biological activities in inflammatory and immunologic responses. These activities are mediated by specific cell surface receptors of 55 kDa and 75 kDa apparent molecular masses. A 75-kDa TNF receptor cDNA was isolated using partial amino acid sequence information and the polymerase chain reaction (PCR). When expressed in COS-1 cells, the cDNA transfers specific TNF-binding properties comparable to those of the native receptor. The predicted extracellular region contains four domains with characteristic cysteine residues highly similar to those of the 55-kDa TNF receptor, the nerve growth factor (NGF) receptor, and the CDw40 and OX40 antigens. The consensus sequence of the TNF receptor extracellular domains also has similarity to the cysteine-rich sequence motif LIM. In marked contrast to the extracellular regions, the intracellular domains of the two TNF receptors are entirely unrelated, suggesting different modes of signaling and function.

Tumor necrosis factor (TNF) is a highly potent cytokine. Its wide range of biological activities in inflammatory and immunologic responses have triggered many studies of the specific cell surface receptors that mediate TNF function.[1-10] TNF receptors of significantly different molecular masses in the range of 50 to 140 kDa were reported in protein cross-linking studies by various investigators; the possibility that more than one receptor existed therefore had to be considered. We have identified and purified from human cell lines and placenta two distinct human TNF receptors of 55 kDa and 75 kDa that are simultaneously expressed to different extents by various cells.[8,11,12] Both receptors bind TNF-α and TNF-β with high affinity[11,13] (also, Schoenfeld and Loetscher, unpublished data). A third TNF-binding protein of 65 kDa was found by SDS-polyacrylamide gel electrophoresis (PAGE) and ligand blotting to copurify with the 75-kDa receptor fraction from HL60 cells. Both the 75-kDa and 65-kDa proteins in Western blots bind the same monoclonal antibody, utr-1.[11] We therefore assume the 65-kDa protein to be a derivative or fragment of the 75-kDa receptor and refer to the two proteins as the 75-kDa receptor.

The cDNA cloning of the 55-kDa receptor has been reported[14,15]; the open reading frame of the cDNA predicts a receptor protein with extracellular, transmembrane, and intracellular regions. A surprisingly high degree of sequence similarity to the nerve growth factor (NGF) receptor extracellular region was discovered which is most clearly delineated by a repetitive cysteine residue pattern. Recently, the cDNA of the 75-kDa TNF receptor was identified in a eukaryotic expression cloning system.[16] We have independently isolated a 75-kDa TNF receptor cDNA using peptide sequencing and PCR techniques which confirms the sequence reported for the cDNA isolated by expression cloning.[16] When expressed in COS-1 cells, the cDNA transfers specific TNF-binding properties comparable to those of the native receptor. The predicted extracellular region contains four domains with characteristic cysteine residues highly similar to that of the 55-kDa TNF receptor and to that of the NGF receptor,[17,18] CDw40,[19] and OX40 antigen[20] extracellular domains. The intracellular domains of the two TNF receptors, however, are entirely unrelated. We therefore propose that the two TNF

[1]Central Research Units, F. Hoffmann-Laroche Ltd, 4002 Basel, Switzerland.
[2]Roche Research Center, Hoffmann-Laroche Inc., Nutley, New Jersey 07110, USA.
*To whom correspondence should be addressed at: Central Research Units, Building 69, Room 14, F. Hoffmann-LaRoche LTD, CH-4002 Basel, Switzerland.

1043-4666/90/0204-0008$05.00/0

KEY WORDS: TNF receptors/Cytokine receptors/NGF-TNF-receptor gene family

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receptors may address distinct intracellular signal transmission pathways.

RESULTS

Isolation of the 75-kDa TNF Receptor cDNA

The 75-kDa and 65-kDa protein bands of the 75-kDa TNF receptor from a preparative SDS-polyacrylamide gel were blotted onto PVDF membrane and subjected to NH_2-terminal amino acid sequencing by gas phase sequenation as reported elsewhere.[12] Briefly, two parallel sequences were obtained with the 65-kDa band; since one sequence matched the ubiquitin sequence, the unique sequence could be identified as LPAQVAFTPYAPEPGSTC.[12] Furthermore, the amino acid sequences of a total of seven internal peptides prepared by tryptic and proteinase K digests of the 75-kDa receptor fraction were determined. The four peptide sequences used in the isolation of the cDNA clone are indicated in Fig. 1; the remaining three peptides, i.e. L^{114}—P^{117}, P^{123}—V^{137} and G^{288}—P^{302}, match the predicted amino acid sequence and thus confirm that the cDNA encodes the receptor. To prepare a probe for the isolation of cDNA clones a short DNA fragment was amplified by polymerase chain reaction (PCR) from human genomic DNA with the use fully degenerate primer oligonucleotides derived from the Q^{412}—L^{428} amino acid sequence (see Fig. 1 and Materials and Methods). A DNA fragment of the predicted size was found to be amplified by PCR. Oligonucleotides were synthesised according to the sequence of this DNA fragment and used to identify the cDNA shown in Fig. 1

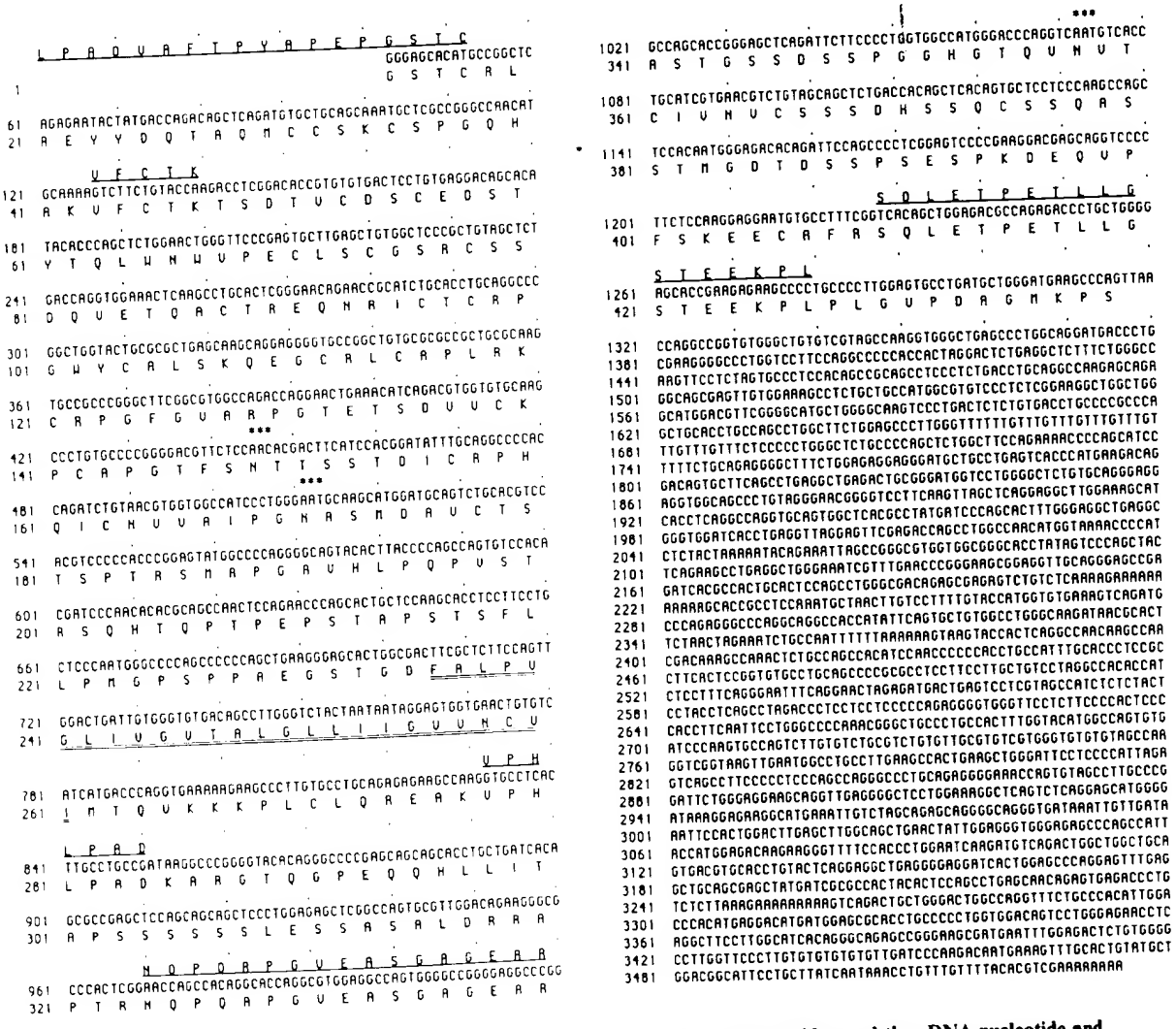

Figure 1. Amino acid sequences of the NH_2 terminus and internal tryptic peptides, and the cDNA nucleotide and predicted amino acid sequences of the 75/65-kDa TNF receptor.

Amino acid sequences determined by protein sequencing are underlined. The amino acid sequence starts at the 65-kDa receptor NH_2 terminus. The predicted transmembrane domain is doubly underlined. Potential N-linked glycosylation sites are marked by asterisks.

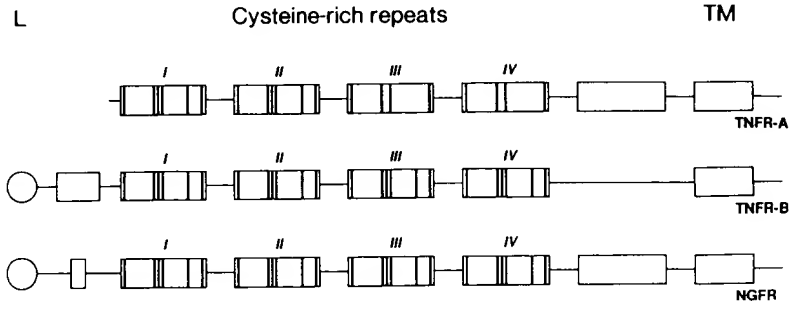

Figure 2. Schematic representation of the domain structure of the extracellular regions of the two TNF receptors and of the NGF receptor.

The domains are boxed. Cysteine residues are represented by vertical lines. The domain boundaries correspond to amino acid residues of Fig. 1: residues 17 to 54 (domain I), 55 to 97 (II), 98 to 140 (III), and 141 to 179 (IV). TNFR-A, 75-kDa TNF receptor; TNFR-B, 55-kDa TNF receptor; NGFR, NGF receptor[17,18]; L and TM, predicted leader and transmembrane regions, respectively.

in cDNA libraries prepared from HL60 and placenta. This cDNA has an open reading frame that predicts a 439-amino acid membrane protein with extracellular (235 residues), transmembrane (26 residues), and intracellular (178 residues) regions. Three basic amino acids are located in the intracellular region sequence adjacent to the putative inner membrane face.

In the predicted amino acid sequence of the extracellular region of the 75-kDa TNF receptor four conserved domains were discovered which are most clearly delineated by a repetitive pattern of cysteine residues schematically represented in Fig. 2. The first two domains contain six cysteine residues in a $CX_{12-14}CX_{0-2}CX_{2-3}CX_{7-9}CX_7C$ pattern which is highly homologous to that of the four domains of the previously reported 55-kDa TNF receptor extracellular region with the consensus sequence $CX_{10-15}CX_{0-2}CX_2CX_{5-11}CX_{3-8}C$.[14,15] In the third and fourth domains of the 75-kDa receptor this cysteine pattern is less well conserved, but the alignment of the total extracellular regions of the two TNF receptors scores significantly above the random score with the Mutation Data Matrix.[21] This alignment score establishes a significant sequence similarity between the extracellular domains of the two TNF receptors as well as to those of the NGF receptor and the CDw40 and OX40 antigens.[17-20] Furthermore, we note that this sequence motif has some similarity to the cysteine-rich, putative metal-binding motif referred to as LIM.[22]

In sharp contrast to the high degree of homology between the extracellular domains, the intracellular regions of the two TNF receptors do not exhibit any recognizable sequence similarity. A search of amino acid sequence data banks with the 75-kDa receptor intracellular domain sequence revealed no significant similarity to other known mammalian sequences. The intracellular regions of both TNF receptors are rich in proline and serine residues (75-kDa receptor: 18% Ser, 9% Pro; 55-kDa receptor: 8% Ser, 12% Pro). Similar proline/serine-rich structures have been found in the intracellular regions of several growth factor receptors.[23,24]

TNF Binding in COS-1 Cell Transfectants

To confirm that the cDNA presented in Fig. 1 encodes a TNF-binding cell surface protein, the cDNA was recloned in the pLJ268 expression vector[25] and transfected into COS-1 cells; transient transfectants were analysed for ^{125}I-TNF binding. Specific TNF-binding properties were conferred to the COS-1 cells by the transfected cDNA (Table 1). Expression of the 75-kDa receptor was confirmed in cell lysates of transfectants with the specific monoclonal antibody utr-4[11] (Table 1). TNF binding was also studied with COS-1 cell transfectants at various ligand concentrations and the binding data were analysed according to Scatchard (Fig. 3). The transfected cells were found to express a TNF-binding protein characterized by a K_d of about 0.1 nM, which is clearly distinct from the endogenous lower-affinity TNF receptor of COS-1 cells.[14] An analysis of the COS-1 cell transfectants in the fluorescence microscope after staining with the 75-kDa TNF receptor–specific monoclonal antibody utr-1 revealed that only a very small percentage of the cells expressed receptor. The cause of the apparently low transfection

TABLE 1. TNF binding and expression of TNF receptor protein in COS-1 cell transfectants

Transfectant	Specific cell surface bound TNF-α		Relative expression of 75/65-kDa versus 55-kDa receptor in cell lysate†
	cpm/dish*	cpm/10^6 cells	
Specific DNA COS-1 cell transfectant	5,170	890	1.39
Control DNA COS-1 cell transfectant 1§	1,230	210	0.05
Control DNA Cos-1 cell transfectant 2§	1,010	185	0.15

*All values are the average of two independent experiments.
‡The quotient of specific 75-kDa and 55-kDa receptor ^{125}I-TNFα binding measured in sandwich assays (see Materials and Methods).
§Controls 1 and 2 refer to parallel transfectants in which constructs in which the cDNA was ligated into the expression vector in a false reading frame were used.

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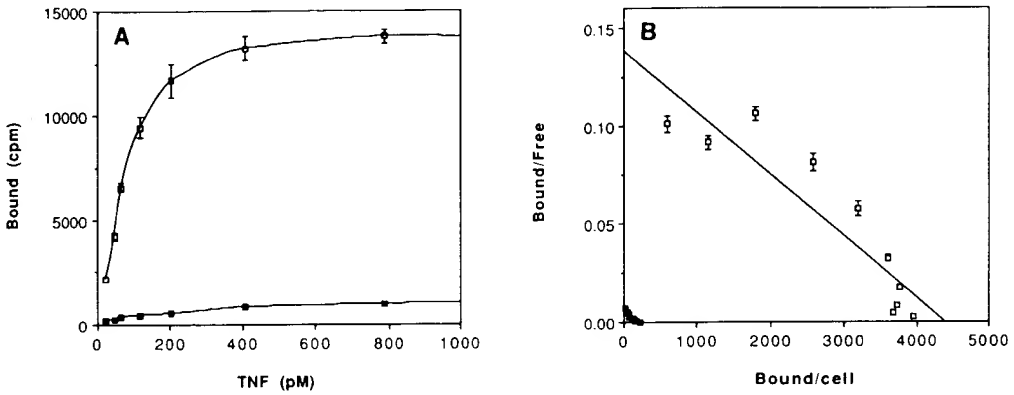

Figure 3. ^{125}I-TNF-α binding to transient COS-1 cell transfectants.

(A) Specific binding at various TNF-α concentrations. Measurements at higher concentrations confirmed saturation of TNF binding (data not included in figure). (B) Plot of the binding data according to Scatchard. The mean and standard deviations of triplicate experiments are given. The assays with transfected and control cells contained 2.2 × 10^6 and 4.3 × 10^6 cells/assay, respectively. □, 75-kDa TNF receptor transfectants; ■, non-transfected control cells. The K_d's of transfected and control cells from Scatchard analysis are about 0.1 and 0.2 nM, respectively.

yield remains unknown, but it explains the low receptor copy number in the pool of transiently transfected cells.

TNF Receptor Expression in Cell Lines

The expression of the TNF receptors was studied in human cell lines by Northern analyses (Fig. 4). Previous flow cytometric analyses of cells stained with receptor-specific monoclonal antibodies had shown that HEp2 cells stain for the 55-kDa receptor only, while HL60 cells stain for both the 55-kDa and 75-kDa receptors.[11] In agreement with previous reports, Raji cells were found to be devoid of TNF receptors.[11,26] These findings were supported by the Northern blot analyses shown in Fig. 4. We note, however, that the lack of 75-kDa TNF receptor expression appears not to be a stable property of Raji cells, since other investigators detect 75-kDa TNF receptor mRNA in these cells.[16] From preliminary studies of 55-kDa and 75-kDa TNF receptor expression HL60 cells appear to be more representative of the average human cell than HEp2 or Raji cells, because many cells were found to express both TNF receptors simultaneously, albeit to very different extents.

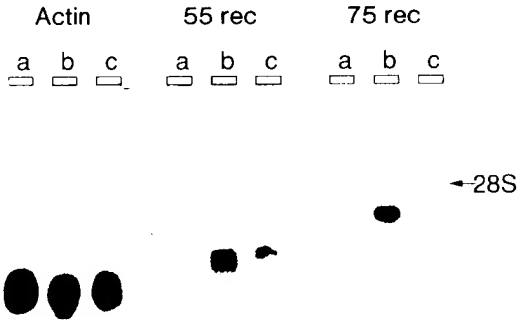

Figure 4. Northern analysis of TNF receptor expression in Raji (a), HL60 (b), and HEp2 (c) cell lines.

By cell surface staining with specific monoclonal antibodies, no TNF receptors are detected on Raji cells, low amounts of 55-kDa receptor (55 rec) are detected on HEp2 cells, and both 55-kDa and 75-kDa receptors (75 rec) are detected at relatively higher levels on HL60 cells.

Expression of Each TNF Receptor is Independently Regulated

To investigate the regulation of the two TNF receptors, we have studied their expression in phytohemagglutinin-activated peripheral blood lymphocytes (PBL). By cell surface staining with the specific monoclonal antibodies utr-1 (anti–75-kDa receptor) and htr-9 (anti–55-kDa receptor),[11] we find that the expression of the 75-kDa receptor is strongly induced from a low resting level, whereas the 55-kDa receptor expression remains at a constant and low level after mitogen activation (Fig. 5). The inducibility of TNF receptors in several cell lines has been previously reported.[27] The finding that the induction is restricted to one type of the two TNF receptors in stimulated PBL as well as analogous findings in cell lines (Hohmann et al., submitted for publication) indicates that the two TNF receptors are functionally distinct.

DISCUSSION

Most human cells express two distinct TNF receptors simultaneously. The molecular cloning of the 55-kDa receptor[14,15] and of the 75-kDa receptor (reference 16 and this work) now allows a comparison of the predicted amino acid sequences of both receptors. The

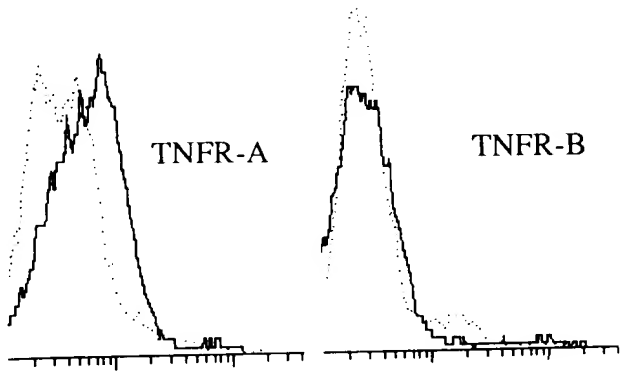

Figure 5. Flow cytometric analysis of 75/65-kDa (TNFR-A) and 55-kDa (TNFR-B) receptor expression on resting (dotted line) and activated (solid line) peripheral blood lymphocytes (PBL).

extracellular regions are found to be highly similar to each other and to those of the NGF receptor and the CDw40 and OX40 antigens. These cell surface molecules thus form a novel gene family. The functional significance of the similarity to the LIM sequence motif[22] remains to be established.

Two TNF-inhibitory peptides of human serum and urine have been described and partial amino acid sequences have been reported.[28-30] One of these inhibitors previously has been recognised as a fragment of the 55-kDa TNF receptor.[14,15] We now find that the short NH_2-terminal sequence of the second inhibitor[30] matches the V^5-P^9 peptide sequence of the 75-kDa TNF receptor (Fig. 1). The Northern blot analysis of cell lines (Fig. 4) reveals a single 75-kDa TNF receptor mRNA species of about 4 kb and provides no evidence for a second message which might encode this inhibitor; analogous conclusions are valid for the other inhibitor.[14] Both of these TNF inhibitory peptides therefore are NH_2-terminally truncated, soluble fragments, presumably of the extracellular regions of the two TNF receptors, and therefore are most likely the products of posttranslational processing of the receptor.

The predicted amino acid sequences of the intracellular regions of the two TNF receptors are unrelated and, furthermore, no similarities to other known mammalian sequences were discovered. It might be concluded that the different intracellular domains transmit distinct signals upon TNF binding to the receptors. However, we cannot presently exclude the possibility that the intracellular regions have no role in signal transduction. A model analogous to that of the interleukin 6 (IL 6) receptor might be considered, where the complex of IL 6 and IL 6 receptor can interact extracellularly with a non–ligand-binding membrane glycoprotein, thus providing the IL 6 signal;[31] both TNF receptors might then address the same signal transducing element. However, in view of the independently regulated expression documented at least in T-cell activation (Fig. 5) it appears more likely that the two TNF receptors are functionally distinct.

MATERIALS AND METHODS

Cells and Flow Cytometry

The cell lines HL60 (ATCC CCL 240), HEp2 (ATCC CCL 23), Raji (ATCC CCL86) and COS-1 (ATCC CRL 1650) were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% inactivated horse or fetal calf serum. Human PBL from a Ficoll gradient were cultured in RPMI 1640, 10% fetal calf serum with or without 2 mg/mL phytohemagglutinin (Wellcome). Cells were stained with biotinylated utr-1 (anti–75-kDa receptor) or htr-9 (anti–55-kDa receptor) antibodies followed by streptavidin-phycoerythrin and analysed on a FACScan flowcytometer.

Reagents

Recombinant human TNF-α purified from *Escherichia coli* was a gift from W. Hunziker, E. Hochuli, and B. Wipf (Hoffmann-LaRoche LTD, Basel). TNF-α was radioiodinated with $Na^{125}I$ (IMS30, Amersham) and Iodo-Gen (Pierce) to 0.3×10^8-1.0×10^8 cpm/μg as described.[32]

cDNA Cloning and Northern Analysis

The 75-kDa and 65-kDa TNF receptors were purified from HL60 cells, and tryptic digests and gas phase sequencing were performed as reported elsewhere.[12] A DNA fragment was prepared from the peptide sequence Q^{412}—L^{428} by PCR on human genomic DNA using 2 low-stringency annealing cycles (95°C 7 min / to 37°C in 2 min / 37°C 1 min / to 72°C in 2.5 min / 72°C 1.5 min / to 95°C in 1 min / 95°C 1 min / to 37°C in 2 min) followed by 38 standard cycles (95°C 1 min / 55°C 2 min / 72°C 2 min); the forward and reverse PCR primers were ctcgaattcCARCTNGARACNCC and ctcgaattcNARNGGYTTYTCYTC, respectively. The DNA band of predicted size from a polyacrylamide gel of the PCR product was recloned, sequenced, and found to encode the Q^{412}—L^{428} peptide. A 48-mer oligonucleotide derived from this DNA was used as a probe to screen cDNA libraries. Several overlapping clones were identified in a human placenta cDNA library in λgt11 (Clontech) and in a HL60 cDNA library in λgt11 that was prepared with the use of cDNA synthesis and cloning kits (Amersham). All recloning and nucleotide sequencing was by standard protocols.[33] For Northern analysis, 12 μg aliquots of Raji-, HL60-, or HEp2-cell total RNA were electrophoresed through an agarose gel containing formaldehyde. RNA was transferred to a Zeta Probe (BioRad) filter, and hybridized to actin, 55-kDa receptor (full length), and 75-kDa receptor (170-bp 5'-fragment) cDNA probes as indicated.

Expression and TNF Binding in COS Cell Transfectants

The cDNA shown in Fig. 1, truncated at the 3'-end was cloned into a pLJ268 vector (gift of B. Cullen[25]) containing the IL 2 receptor signal sequence under the control of the RSV long terminal repeat promoter and polyadenylation signals

derived from the rat preproinsulin II genomic gene. DNA was transiently transfected into COS-1 cells with DEAE dextran following standard protocols.[33] Specific ^{125}I-TNF-α binding on transfectants was measured in the absence and presence of excess unlabeled TNF-α after 3 days in culture as previously reported[14] and Scatchard analysis was carried out. Briefly, COS-1 cells were detached with EDTA (GIBCO), washed and incubated with ^{125}I-TNF-α for 2 hr at 4°C; cell-bound and free radioactivity was then counted. Aliquots of transfected cells were lysed by 1.0% Triton X-100. The expression of the 75-kDa TNF receptor and of the "55-kDa-type" endogenous COS-1 cell receptor was measured in transfectant cell lysates in a solid phase sandwich assay using the 75-kDa and 55-kDa receptor-specific monoclonal antibodies utr-4 and htr-20, respectively, and with ^{125}I-TNF-α in the absence and presence of unlabeled TNF-α. The relative receptor expression in the cell lysate in Table 1 is defined as the quotient of specific 75-kDa and 55-kDa TNF receptor ^{125}I-TNF-α binding measured in the two sandwich assays. Controls 1 and 2 refer to parallel transfectants in which constructs where the cDNA was ligated into the expression vector in a false reading frame were used.

Acknowledgments

We thank M. Steinmetz for stimulating discussions, W. Bannwarth for synthetic oligonucleotides, and W. Eufe, N. Grau, A. Hayes, J.D. Hulmes, C. Kocyba, C. Kuerschner, H.P. Kurt, K. McCune, M. Ott, U. Roethlisberger and L. Stehrenberger for help and excellent technical support.

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AU   Hsu H; Xiong J; Goeddel D V
TI   The   ***TNF***   receptor 1-associated protein TRADD signals cell
     death and NF-kappa B activation.
SO   CELL, (1995 May 19) 81 (4) 495-504.
     Journal code: CQ4. ISSN: 0092-8674.
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Cell, Vol. 81, 495–504, May 19, 1995

The TNF Receptor 1-Associated Protein TRADD Signals Cell Death and NF-κB Activation

Hailing Hsu, Jessie Xiong, and David V. Goeddel
Tularik, Incorporated
270 East Grand Avenue
South San Francisco, California 94080

Summary

Many diverse activities of tumor necrosis factor (TNF) are signaled through TNF receptor 1 (TNFR1). We have identified a novel 34 kDa protein, designated TRADD, that specifically interacts with an intracellular domain of TNFR1 known to be essential for mediating programmed cell death. Overexpression of TRADD leads to two major TNF-induced responses, apoptosis and activation of NF-κB. The C-terminal 118 amino acids of TRADD are sufficient to trigger both of these activities and likewise sufficient for interaction with the death domain of TNFR1. TRADD-mediated cell death can be suppressed by the *crmA* gene, which encodes a specific inhibitor of the interleukin-1β-converting enzyme. However, NF-κB activation by TRADD is not inhibited by *crmA* expression, demonstrating that the signaling pathways for TNF-induced cell death and NF-κB activation are distinct.

Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine whose varied biological activities are signaled through two distinct cell surface receptors (reviewed by Tartaglia and Goeddel, 1992). These receptors, termed TNFR1 and TNFR2, are of approximate masses 55 kDa and 75 kDa, respectively, and are expressed on most cell types (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). The primary amino acid sequences of the intracellular domains bear no detectable similarity, leading to the prediction that the two receptors interact with different proteins and activate distinct signal transduction pathways (Lewis et al., 1991). Gene knockout experiments (Pfeffer et al., 1993; Rothe et al., 1993; Erickson et al., 1994) and studies with receptor-specific agonistic antibodies (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992; Gehr et al., 1992) have confirmed this prediction and demonstrated that the two TNF receptors generate largely nonoverlapping signals. The majority of the known activities of TNF have been attributed to TNFR1. Direct signaling through TNFR2 occurs less extensively and appears to be mainly confined to cells of the immune system.

The two TNF receptors are members of the growing TNF receptor superfamily, which includes the Fas antigen and CD40 (reviewed by Smith et al., 1994). The binding to these receptors of their respective ligands induces receptor oligomerization and is thought to link receptors to downstream signaling pathways (reviewed by Tartaglia and Goeddel, 1992; Smith et al., 1994). However, direct coupling to such pathways has not yet been demonstrated. A potential breakthrough in this regard was the identification of two related proteins, TRAF1 and TRAF2 (for TNF receptor–associated factors 1 and 2, respectively), that form a heterodimeric complex and associate with the cytoplasmic domain of TNFR2 (Rothe et al., 1994). TRAF1 and TRAF2 share a novel region of homology, the TRAF domain. A third member of this protein family, designated CD40bp or TRAF3, has recently been shown to associate with the cytoplasmic domain of CD40 (Hu et al., 1994). However, the actual contributions of the TRAFs to signal transduction by TNFR2 and CD40 remain unresolved.

TNFR1 is responsible for most of the biological properties of TNF, including programmed cell death, antiviral activity, and activation of the transcription factor NF-κB in a wide variety of cell types (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992; Tartaglia et al., 1993). It also plays an essential role in host defense against microorganisms and bacterial pathogens (Pfeffer et al., 1993; Rothe et al., 1993). Mutagenesis studies have identified a so-called death domain of approximately 80 amino acids near the C-terminus of TNFR1 that is required for signaling antiviral activity and cell death (Tartaglia et al., 1993) as well as for NF-κB activation (Y.-F. Hu and D. V. G., unpublished data). The death domain of TNFR1 also triggers activation of an endosomal acidic sphingomyelinase (Wiegmann et al., 1994). A homologous domain that can also initiate programmed cell death is found in the Fas antigen (Itoh and Nagata, 1993). The apoptosis induced by both TNF and Fas was recently shown to involve the activation of the interleukin-1β-converting enzyme (ICE) or an ICE-like cysteine protease (Tewari and Dixit, 1995).

The TNFR1-associated proteins involved in generating the various TNF-induced signals remain unknown, although coimmunoprecipitation experiments have revealed that three phosphoproteins and a serine protein kinase activity associate with TNFR1 following TNF treatment (VanArsdale and Ware, 1994). In this report, we describe the molecular cloning of TNFR1-associated death domain protein (TRADD), a novel protein that interacts specifically with the death domain of TNFR1. Overexpression of TRADD activates two major TNF signaling pathways, apoptosis, and NF-κB activation. Furthermore, the ICE inhibitor encoded by the cowpox virus *crmA* gene protects against TRADD-mediated cell death. However, crmA does not prevent TRADD-induced NF-κB activation, demonstrating that the two signaling pathways emanating from TRADD are distinct.

Results

Isolation of cDNA Clones Encoding TNFR1-Interacting Proteins

To identify proteins that directly interact with the intracellular region of human TNFR1, we used the yeast two-hybrid system (Fields and Song, 1989). From approximately 50

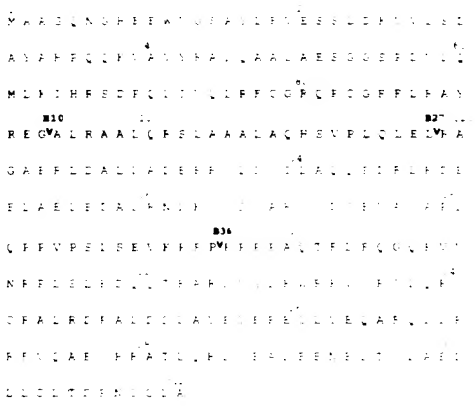

Figure 1. Predicted Amino Acid Sequence of TRADD

The amino acid sequence deduced from the sequence of two full-length *TRADD* cDNAs is shown. The three clones isolated by two-hybrid screening were fused to Gal4ad at the positions indicated by H10, B27, and B36.

million transformants, 48 positive clones, as determined by activation of *his* and *lacZ* reporter genes, were obtained. Of these clones, 41 encoded portions of the cytoplasmic region of TNFR1, indicating that the death domain (Tartaglia et al., 1993) of the receptor can self-associate. Similar findings using the yeast two-hybrid system were reported recently (Song et al., 1994; Boldin et al., 1995). Three of the remaining seven clones (H10 [219 amino acids], B27 [194 amino acids], and B36 [118 amino acids]) were partial-length cDNAs derived from the same gene. We screened human umbilical vein endothelial cell (HUVEC) and HeLa cDNA libraries by using the B27 cDNA as probe and obtained four cDNAs of approximately 1.5 kb. DNA sequence analysis of these clones revealed an open reading frame predicted to encode a protein of 312 amino acids (Figure 1) with a molecular mass of 34.2 kDa that we have designated TRADD. Database searches utilizing BLAST and FASTA programs failed to identify any proteins having significant sequence similarity to TRADD.

Detection of *TRADD* mRNA and Protein

Northern blot analysis indicated that low amounts of *TRADD* mRNA were expressed constitutively in all human tissues examined (Figure 2A). This result is consistent with TRADD involvement in TNFR1 signal transduction, as TNFR1 mRNA is also expressed ubiquitously (Loetscher et al., 1990; Schall et al., 1990; Lewis et al., 1991). The ~1.4 kb size of the *TRADD* transcript confirms that the cDNA clones represent full-length copies of *TRADD* mRNA.

Polyclonal antibodies against TRADD were generated by expressing a glutathione S-transferase (GST)–TRADD fusion protein in Escherichia coli and using the purified GST–TRADD chimera as immunogen. Rabbit anti-TRADD antiserum specifically recognized two proteins of approximately 34 kDa when tested by Western blot analysis using lysates prepared from 293 cells transiently transfected with a *TRADD* expression vector (Figure 2B). The weak upper band of the doublet may be a posttranslationally modified TRADD. Protein bands of the same size, corresponding to endogenous TRADD, could be detected in lysates from ECV304, HeLa, and HepG2 cells (Figure 2B) prepared with 50-fold more cells.

TRADD Specifically Interacts with TNFR1 and Self-Associates

To confirm that full-length TRADD interacts specifically with TNFR1, a protein consisting of the GAL4 activation domain fused to full-length TRADD (GAL4ad–TRADD) was coexpressed with the GAL4bd–TNF–R1icd fusion protein in yeast strain SFY526. The GAL4ad–TRADD chimera interacted with the GAL4bd–TNF–R1icd fusion protein, but not with the GAL4 DNA-binding domain alone (Table 1). We also tested the ability of the GAL4ad–TRADD fusion protein to interact with receptors that are related to TNFR1 either in terms of their structure (TNFR2 and Fas antigen) or the signals they generate (Fas antigen and the type I interleukin-1 receptor [IL-1R1]). GAL4ad–TRADD failed to interact with the cytoplasmic domains of TNFR2, IL-1R1, or Fas antigen expressed as GAL4bd chimeras (Table 1).

To examine further the specificity of the interaction between TRADD and TNFR1, the GAL4bd was fused to two different C-terminal deletion mutants of TNF–R1icd as well as to the cytoplasmic domain of murine TNFR1. GAL4ad–TRADD interacted strongly with an active receptor mutant (Δ413–426) lacking 14 amino acids, yet interacted weakly with an inactive mutant (Δ407–426) lacking 20 amino acids (Table 1). These results indicate that amino acids 407–412 of TNFR1, which are required for the signaling of cell death (Tartaglia et al., 1993), contribute to, but are not required for, its TRADD interaction. TRADD also interacted with mouse TNFR1 in this assay system, but more weakly than with the homologous human TNFR1.

We performed in vitro biochemical assays to confirm the specific interaction of TNFR1 with TRADD observed in the two-hybrid system. GST fusion proteins containing the cytoplasmic domains of TNFR1, TNFR2, IL-1R1, and Fas antigen were tested for interaction with ^{35}S-labeled TRADD prepared by in vitro transcription and translation. TRADD associated only with the GST–TNFR1 fusion protein (Figure 3A). Furthermore, TRADD did not associate with a GST–TNFR1(–20) fusion protein derived from the inactive TNFR1 Δ407–426 mutant.

To determine whether TRADD exists as a monomer, we prepared a reverse two-hybrid construct in which TRADD was fused to the GAL4bd. The results of a yeast cotransformation experiment using this construct and the GAL4ad–TRADD construct indicated that these two proteins interact with each other (Table 1). The simplest interpretation of this result is that TRADD is an oligomeric protein.

TRADD–TNFR1 Interaction in Human Cells

Extensive mutational analysis of TNFR1 has led to the identification of several residues in its death domain that are important for signaling cell death (Tartaglia et al., 1993). To determine whether a correlation exists between

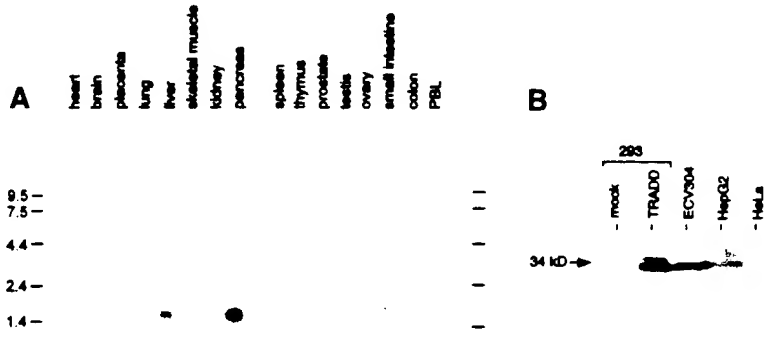

Figure 2. Identification of *TRADD* mRNA and Protein

(A) Northern blot analysis of *TRADD* mRNA in multiple human tissues. *TRADD* mRNA is the band at ~1.4–1.5 kb. The weakly hybridizing band at ~5 kb is from nonspecific hybridization to 28S rRNA.

(B) Western blot analysis of TRADD protein in mock- and pRK-TRADD-transfected 293 cells, and in nontransfected ECV304, HepG2, and HeLa cells. Lysates were prepared from 2×10^4 293 cells (lanes 1–2) or 10^6 ECV304, HepG2, and HeLa cells (lanes 3–5) and proteins separated by SDS–PAGE on a 10% gel. Western blotting was performed with rabbit anti-TRADD antiserum. Positions of molecular weight standards (in kilodaltons) are shown on the left. An equivalent Western blot using preimmune serum failed to detect any proteins in the 34 kDa range (data not shown).

the ability of a TNFR1 mutant to deliver a cytotoxic signal and to interact with TRADD, we utilized a mammalian cell coimmunoprecipitation assay. An expression vector that directs the synthesis of TRADD containing an N-terminal Myc epitope tag was cotransfected with various TNFR1 constructs into human embryonic kidney 293 cells. Cell xtracts were immunoprecipitated by using polyclonal antibodies against the extracellular domain of TNFR1, and coprecipitating TRADD was detected by Western blotting with an anti-Myc monoclonal antibody. As determined by this assay, TRADD specifically associates with TNFR1 (Figure 3B). Five different deletion and point mutants of TNFR1 were also examined by this method. The two active mutants (Δ413–426 and Δ212–308) were able to coprecipitate TRADD, whereas two of the three inactive mutants (Δ212–340 and K343, F345, R347) failed to do so. The third inactive mutant (Δ407–426) coprecipitated TRADD w akly in some experiments (Figure 3B) and not at all in others (data not shown). Accordingly, it appears that r sidues throughout the ~80 amino acid death domain of TNFR1 are critical for TRADD interaction.

Overexpression of TRADD Induces Apoptosis

One of the major activities signaled by TNF through TNFR1 is programmed cell death or apoptosis. To investigate a possible role for TRADD in TNF-mediated apoptosis, 293 cells were transiently transfected with a *TRADD* expression vector and examined by phase contrast microscopy 24 hr later. Those 293 cells that overexpressed TRADD had obvious morphological differences from those transfected with a control vector (Figure 4A). The TRADD-expressing cells displayed the typical characteristics of adherent cells undergoing apoptosis, becoming rounded and condensed (average diameter ~15 μm) and detaching from the dish. Cells transfected with a control vector remained flat, with an average length of ~40–50 μm.

One biochemical hallmark of apoptosis is the internucleosomal fragmentation of nuclear DNA, which results in a distinct laddering pattern when analyzed by gel electrophoresis (Tomei and Cope, 1991). We examined nuclear DNA from 293 cells transfected with either a contol vector or with the *TRADD* expression vector. DNA isolated from

Table 1. Interactions between TRADD and TNFR1

DNA-Binding Hybrid	Activation Hybrid	Colony Color	Relative β-Galactosidase Activity
GAL4bd-TRADD	GAL4ad	White	<1
GAL4bd	GAL4ad-TRADD	White	<1
GAL4bd-TRADD	GAL4ad-TRADD	Blue	69
GAL4bd-TNFR1	GAL4ad-TRADD	Blue	47
GAL4bd-TNFR1(-14)	GAL4ad-TRADD	Blue	76
GAL4bd-TNFR1(-20)	GAL4ad-TRADD	Light blue	12
GAL4bd-muTNFR1	GAL4bd-TRADD	Light blue	27
GAL4bd-IL1R1	GAL4ad-TRADD	White	<1
GAL4bd-Fas	GAL4ad-TRADD	White	<1
GAL4bd-TNFR2	GAL4ad-TRADD	White	<1

Yeast SFY526 cells were cotransformed with expression vectors encoding various GAL4 DNA-binding domain (GAL4bd) and GAL4 transcription activation domain (GAL4ad) fusion proteins. Each transformation mixture was plated on two synthetic dextrose plates lacking tryptophan and leucine. One plate was used to perform filter assays for colony color. Colonies on the second plate from each transformation were combined (at least 100 colonies per plate) and grown in liquid culture. β-Galactosidase activity was determined on the pooled colonies by the CPRG assay (Iwabuchi et al., 1993).

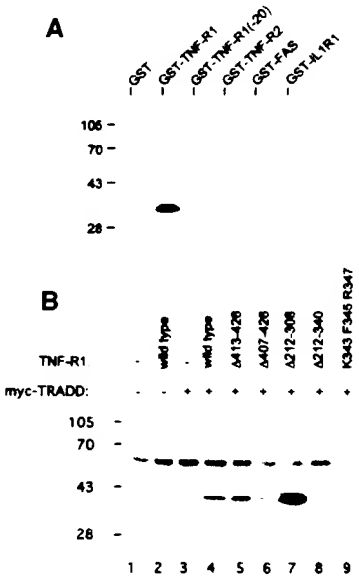

Figure 3. Interaction of TRADD with TNFR1

(A) In vitro interaction of ^{35}S–TRADD with the intracellular regions of several receptors expressed as GST fusion proteins. ^{35}S–TRADD was incubated with purified GST fusion proteins and processed as described in Experimental Procedures. Positions of molecular weight standards (in kilodaltons) are shown.

(B) In vivo interaction of TRADD with TNFR1 mutants. 293 cells (2 × 10^5) were transiently transfected with the Myc epitope–tagged *TRADD* expression vector (1 μg) and the indicated pRK–TNFR1 constructs (1 μg). After 24 hr, extracts were prepared and immunoprecipitated with polyclonal antibody to TNFR1. Coprecipitating Myc–TRADD was detected by immunoblot analysis using the anti-Myc monoclonal antibody.

Table 2. Cell Death Induced by TRADD Overexpression

Expression Vector	Number of Blue Cells per Well		
	HeLa	HepG2	NIH 3T3
pRK control	2110 ± 195	214 ± 16	608 ± 34
TRADD	25 ± 3	1 ± 1	1 ± 1
TRADD, crmA	2083 ± 61	66 ± 20	625 ± 17
TRADD, Bcl-2	1151 ± 74	0 ± 0	21 ± 3
TRADD, E1B	75 ± 9	0 ± 0	5 ± 2

The indicated cell lines were transiently cotransfected with pRK-TRADD (1 μg), pCMV-βgal (0.5 μg), and 3.5 μg of expression vector for *crmA*, *Bcl-2*, or E1B into the various cell lines (2 × 10^5 cells/well). Samples were supplemented with the pRK5 vector control to bring total DNA for each transfection to 5 μg. Cells were fixed and stained with X-Gal 36 hr after transfection. Data (± SEM) are shown as the number of blue cells per 35 mm dish for at least three independent transfections.

TRADD-expressing cells displayed a profile characteristic of apoptosis and closely resembled the DNA from untransfected 293 cells that were treated with TNF (Figure 4B).

It was recently shown (Tewari and Dixit, 1995) that TNF-induced apoptosis can be inhibited by the ICE-specific serpin inhibitor encoded by the cowpox *crmA* gene (Ray et al., 1992). This led us to examine the effects of crmA protein and other known inhibitors of apoptosis (Bcl-2 and adenovirus 19K E1B proteins) on TRADD-induced cell death. The appearance of 293 cells cotransfected with expression vectors encoding both TRADD and crmA was indistinguishable from cells transfected with a control vector alone (Figure 4A). Conversely, coexpression of either the *Bcl-2* or *E1B* gene product did not counteract the apoptotic effect of TRADD expression on 293 cells. Furthermore, crmA coexpression also blocked the generation of TRADD-induced DNA laddering (Figure 4B). These results are consistent with the interpretation that TRADD-induced apoptosis involves activation of ICE or a related protease.

To ensure that the induction of apoptosis by TRADD was not a peculiarity of 293 cells, we also examined the effects of TRADD overexpression on HeLa, HepG2, and murine NIH 3T3 cells. In these cases, a β-galactosidase cotransfection assay (Kumar et al., 1994) was used to examine cell viability. Cells were transiently transfected with a β-galactosidase expression plasmid and the various expression vectors described above. After 36 hr, cells were stained with X-Gal and positive blue cells visualized and

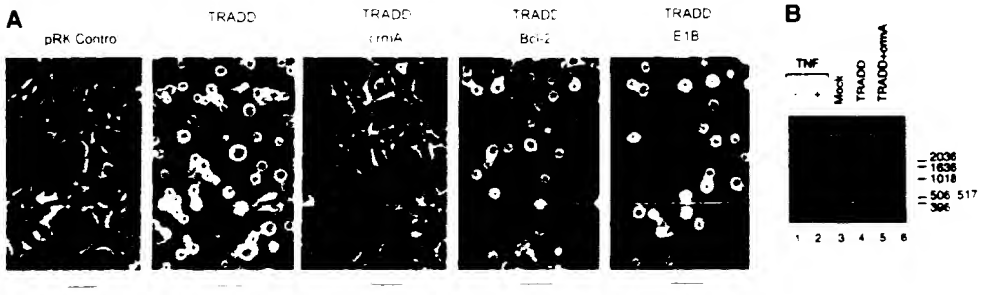

Figure 4. Apoptosis Induced by TRADD Overexpression and Its Inhibition by crmA Expression

(A) Morphology of 293 cells transiently overexpressing TRADD. Cells (2 × 10^5) were transiently transfected with the indicated expression vectors (1 μg) and analyzed 24 hr later by phase contrast microscopy for signs of apoptosis. Scale bar, 50 μm.

(B) DNA fragmentation in transfected 293 cells and TNF-treated 293 cells. Untransfected 293 cells (2 × 10^6) were treated with 320 nM actinomycin D in the presence or absense of TNF (100 ng/ml) for 18 hr before preparation of genomic DNA. Large-scale transfections of 2 × 10^6 293 cells were performed with the control vector pRK5 (20 μg), pRK–TRADD (5 μg) plus pRK5 (15 μg), or pRK–TRADD (5 μg) plus pRK–crmA (15 μg). After 24 hr, DNA was isolated and analyzed as described in Experimental Procedures. The positions of the size markers (in base pairs) are shown on the right.

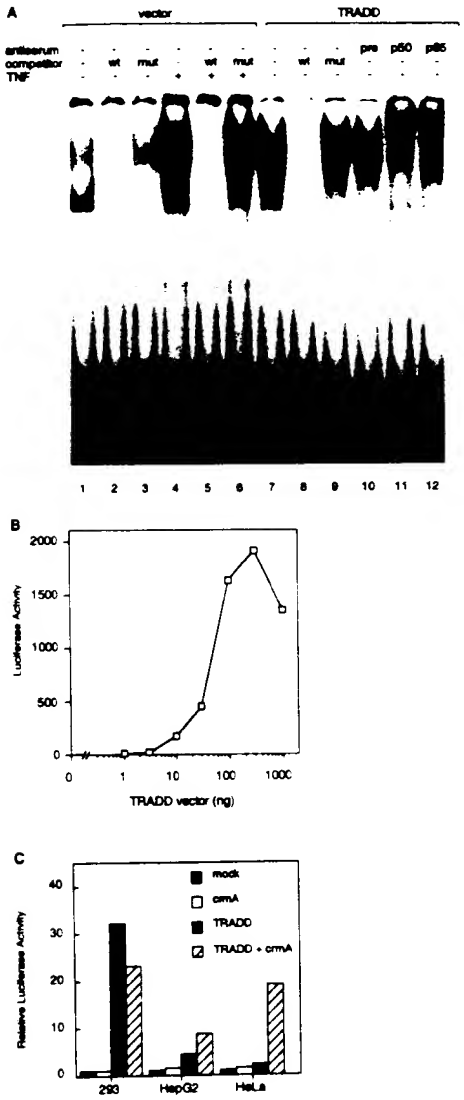

Figure 5. Transcription Factor NF-κB is Activated by TRADD Overexpression

(A) EMSA of NF-κB activation in 293 cells. 293 cells (10^6) were transfected with 10 μg of pRK5 control (lanes 1–6) or pRK–TRADD (lanes 7–12) DNA. Cells were treated with 100 ng/ml TNF for 3 hr prior to harvest (lanes 4–6). Nuclear extracts were prepared 24 hr after transfection, and 10 μg aliquots were combined with the ^{32}P-labeled NF-κB oligonucleotide probe. Individual reactions were supplemented with a 50-fold excess of cold competitor oligonucleotide containing either a wild-type (lanes 2, 5, 8) or mutated (lanes 3, 6, 9) NF-κB sequence. Reaction mixtures were incubated with 1 μl of preimmune serum (lane 10), anti-p50 serum (lane 11), or anti-p65 serum (lane 12) for 10 min prior to addition of probe.

(B) Effect of TRADD expression on NF-κB activity in 293 cells determined by an E-selectin promoter–luciferase gene reporter assay. 293 cells (2×10^5) were transfected with 1 μg of pELAM-luc reporter plasmid, 0.5 μg of pRSV-βgal, indicated amounts of pRK–TRADD, and enough pRK5 control plasmid to give 2.5 μg of total DNA. Luciferase activities were determined 24 hrs after transfection and normalized on the basis of β-galactosidase expression levels. Values shown are averages for an experiment in which each transfection was performed in duplicate.

(C) NF-κB activation by TRADD overexpression in 293, HepG2, and HeLa cells. Cells (2×10^5) were transfected with 4 μg of total DNA as follows. Each transfection received 1 μg of pELAM-luc, 0.5 μg of pRSV-βgal, and one of each of the following: 2.5 μg of pRK5 control vector (solid black bars); 2 μg of pRK–crmA, 0.5 μg of pRK5 (solid white bars); 0.5 μg of pRK–TRADD, 2 μg of pRK5 (dark shading); or 0.5 μg of pRK–TRADD, 2 μg of pRK–crmA (light shading). Cells were harvested 24 hr after transfection and luciferase levels determined. The fold induction in luciferase activity compared with the control pRK5 transfection is indicated.

counted. In all cell lines, a dramatic (~100-fold) reduction in the number of β-galactosidase-positive cells was observed for the *TRADD* vector compared with the control vector (Table 2). When crmA and TRADD were coexpressed, the numbers of blue HeLa and NIH 3T3 cells were the same as in the vector controls. Coexpression of crmA protected about one-third of transfected HepG2 cells from TRADD-induced cell death. As was seen in 293 cells, neither Bcl-2 nor E1B expression exerted a protective effect on TRADD-mediated apoptosis in HeLa or NIH 3T3 cells. Bcl-2 did provide partial protection for HeLa cells from the effect of TRADD overexpression (Table 2).

TRADD Overexpression Activates NF-κB

Another important activity of TNF signaled by TNFR1 is activation of the transcription factor NF-κB. To examine a possible role for TRADD in this process, we performed electrophoretic mobility shift assays (EMSAs) on nuclear extracts from transfected 293 cells. TRADD-expressing 293 cells were found to contain a significant amount of activated NF-κB even in the absense of exogenous TNF. In contrast, specific NF-κB complexes were detected only after TNF treatment in 293 cells transfected with an empty expression vector (Figure 5A). Supershift experiments performed with antibodies demonstrate that the major component of the activated NF-κB complex appears to be the p65–p50 heterodimer.

Dose–response experiments were performed to determine whether TRADD expression might lead to activation of a NF-κB-dependent reporter gene. An E-selectin–luciferase reporter construct was cotransfected with increasing amounts of the *TRADD* expression vector into 293 cells. TRADD expression potently activated the reporter gene, with maximal luciferase activity (approximately 400-fold induction) occurring at a 0.32 μg dose of the *TRADD* expression vector (Figure 5B). These levels of reporter gene induction are greater than the ~20-fold induction observed when 293 cells are treated with TNF alone (data not shown). Further increases in *TRADD* vector resulted in diminished levels of reporter activity, probably owing to induction of cell death (see below).

CrmA Does Not Block TRADD-Induced NF-κB Activation

Since crmA is a potent inhibitor of apoptosis induced by either TNF treatment or TRADD expression, we examined its effect on NF-κB activation. CrmA expression had little or no effect on TRADD-induced NF-κB activation in 293 cells as determined either by EMSA (data not shown) or by the luciferase reporter gene assay (Figure 5C). HeLa and HepG2 cells were also examined for NF-κB activation following transient transfection with th *TRADD* expr s-

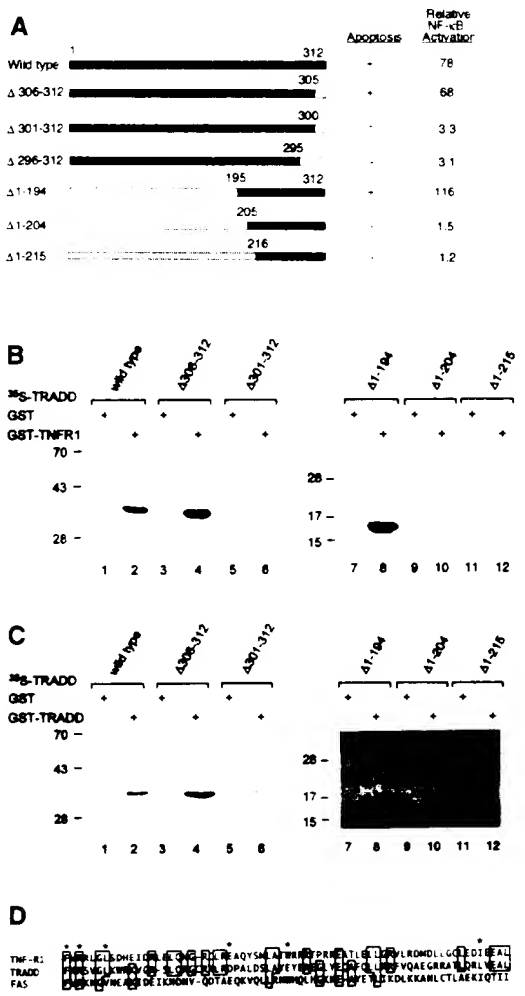

Figure 6. Analysis of TRADD Deletion Mutants

(A) Apoptosis and activation of NF-κB mediated by TRADD deletion mutants. The horizontal bars represent the sequence of TRADD, with shaded regions corresponding to intact sequences and dotted regions indicating deleted sequences. Apoptosis assays were performed 24 hr after transfection of 293 cells with the indicated Myc-tagged TRADD constructs. Plus indicates that the majority of cells became condensed, rounded, and detached (as in Figure 4A), while minus indicates that no morphological changes were observed. NF-κB activation assays were performed following cotransfection of 293 cells with TRADD and the NF-κB reporter plasmid pELAM-luc as described in Experimental Procedures. Data are shown as fold increase in luciferase levels relative to levels in 293 cells transfected with the control vector pRK5.

(B) Interaction of ^{35}S-TRADD deletion mutants with GST-TNFR1. The various ^{35}S-labeled TRADD deletion mutants (equivalent counts per minute) were incubated with purified GST or GST-TNFR1 bound to glutathione-Sepharose beads and processed as described in Experimental Procedures. C-terminal (lanes 1–6) and N-terminal (lanes 7–12) deletion mutants were fractionated on 10% and 15% SDS-PAGE, respectively, and exposed to X-ray film for 8 hr.

(C) In vitro interaction of ^{35}S-TRADD deletion mutants with GST-TRADD. Experiments were performed as described above but using GST-TRADD instead of GST-TNFR1. Dried gels were exposed to X-ray film for 5 days.

(D) Alignment of TRADD, TNFR1, and Fas death domains. Amino acids 222-289 of TRADD are compared with amino acids 345–412 of TNFR1 and 232–298 of Fas antigen. Boxes indicate identities between TRADD and either TNFR1 or Fas. Asterisks above the sequence indicate the six individual residues where mutation to alanine has been shown to inactivate TNFR1 signaling (Tartaglia et al., 1993).

sion vector alone. In most experiments, no activation was seen in these cell lines, a result potentially attributable to rapid induction of cell death (see Table 2). To determine whether NF-κB could be activated by TRADD if cell death were inhibited, these cell lines were cotransfected with *crmA* and *TRADD* expression vectors. While crmA expression alone had no effect, substantial activation of NF-κB was observed in the *crmA–TRADD* cotransfection experiments (Figure 5C). These results demonstrate an inherent ability of TRADD to activate NF-κB, which can be more readily observed if the death pathway is blockaded by crmA expression.

Deletion Mutagenesis of TRADD

The experiments described thus far have identified four distinct properties of TRADD: first, interaction with TNFR1; second, self-association; third, induction of apoptosis; and fourth, activation of NF-κB. To ascertain whether these properties reside in common or distinct domains of TRADD, we constructed a series of N- and C-terminal deletion mutants (Figure 6A). The ability of each mutant to induce apoptosis was determined in the 293 transient assays described above. A TRADD mutant (Δ1–194) containing only 118 C-terminal amino acids was able to trigger cell death, whereas a mutant (Δ1–204) ten amino acids shorter did not. Deleting from the C-terminus, a mutant (Δ301–312) lacking only 12 residues was inactive, but a mutant (Δ306–312) lacking seven amino acids retained the ability to induce apoptosis of 293 cells. This analysis localized the apoptosis activation function (death domain) of TRADD to a 111 residue region extending from amino acid 195 to amino acid 305 (Figure 6A). NF-κB activation for each TRADD mutant was determined in the NF-κB reporter cotransfection assay. According to this analysis, the region of TRADD required for NF-κB activation was strictly concordant with that required for apoptosis (Figure 6A).

The ^{35}S-labeled TRADD deletion mutants were assayed for association with a GST–TNFR1 fusion protein (Figure 6B). Two mutants (Δ306–312 and Δ1–194) bound as well as wild-type TRADD to TNFR1. These same two mutants were biologically active, as determined by the apoptosis and NF-κB activation assays. TRADD mutants that were inactive in these biological assays bound TNFR1 poorly or not at all.

Similarly, the ability of the TRADD deletion mutants to self-oligomerize was assessed by use of a GST–TRADD fusion protein and the ^{35}S-labeled TRADD mutants (Figure 6C). The same mutants that interacted with GST–TNFR1 were able to bind GST–TRADD, although by this assay, self-association was much weaker than interaction with TNFR1. The one difference observed was that the biologically inactive Δ301–312 mutant, which does not interact with TNFR1, was still able to self-associate. These results demonstrate that a 111 amino acid domain (amino acids 195–305) of TRADD is capable of oligomerization, TNFR1 interaction, stimulation of programmed cell death, and NF-κB activation.

Death Domains of TRADD and TNFR1 Share Sequence Similarity

Database screens failed to identify any proteins bearing significant similarity to the primary amino acid sequence of TRADD. However, since the 111 amino acid death domain of TRADD shares many properties with the ~80 amino acid death domain of TNFR1, these two sequences were directly compared. Alignment of TRADD residues 196–302 relative to residues 319–425 of TNFR1 results in 25 identities over 107 amino acids (23%). The most obvious sequence similarity was found in the stretch of 68 amino acids shown in Figure 6D. If a single gap is introduced into both sequences, 22 identities (32%) and 21 conservative changes are found. Whereas the majority of the identities are leucines and arginines, three observations indicate that the sequence similarity may be functionally significant. First, six amino acids have been identified in the death domain of TNFR1 that, when mutated to alanine, abolish signaling (Tartaglia et al., 1993). Five of these six amino acids, which extend to both ends of the aligned sequence (Figure 6D), are identical or highly conserved in TRADD. Second, the sequence similarity between TRADD and TNFR1 over this region is roughly the same as that between the functionally similar death domains of TNFR1 and Fas antigen. Third, TRADD and Fas antigen, which appear not to interact with each other, share only nine identities (13%) over these 68 residues.

Discussion

TRADD Activates TNFR1 Signaling Pathways

The signal transduction events initiated by ligand binding to receptor tyrosine kinases (Schlessinger and Ullrich, 1992) and hematopoietic cytokine receptors (Kishimoto et al., 1994) are now relatively well understood. In contrast, little is known at the molecular level about the postreceptor signaling mechanisms utilized by the TNF receptor superfamily. There are now twelve known members of this family, yet unambiguous evidence for direct receptor coupling with intracellular proteins exists only for TNFR2 and CD40. In the former instance, a heterodimer consisting of the two related proteins TRAF1 and TRAF2 associates via TRAF2 with sequences in the TNFR2 cytoplasmic domain that are necessary for signal transduction (Rothe et al., 1994). In the latter case, another TRAF domain protein, CD40bp or TRAF3, is found to associate with CD40 (Hu et al., 1994).

Most of the known pleiotropic activities of TNF are a consequence of TNFR1 activation (Tartaglia and Goeddel, 1992). An ~80 amino acid death domain located near the C-terminus of TNFR1 is sufficient to initiate signals for apoptosis, antiviral activity, and NF-κB activation (Tartaglia et al., 1993; Y.-F. Hu and D. V. G., unpublished data). To identify candidate proteins for evaluation as TNFR1-associated signaling molecules, we utilized the yeast two-hybrid cloning approach of Fields and Song (1989). Consistent with the work of others (Song et al., 1994; Boldin et al., 1995), we found that the cDNAs isolated most frequently were those encoding the death domain of TNFR1. We also identified TRADD, a 34 kDa protein bearing little resemblance to previously described proteins.

Overexpression of TRADD in a variety of cell lines was found to be a potent inducer of programmed cell death, mimicking the effect of TNF treatment in the presence of actinomycin D. TRADD-mediated apoptosis is effectively blocked by coexpression of the cowpox virus *crmA* gene, which encodes a protease inhibitor of the serpin class (Pickup et al., 1986). CrmA is a specific inhibitor of ICE (Ray et al., 1992), a cysteine protease involved in IL-1β processing (Thornberry et al., 1992) and in some types of programmed cell death (Miura et al., 1993; Gagliardini et al., 1994). Significantly, crmA was recently shown to block the apoptosis triggered by both TNFR1 and Fas antigen (Tewari and Dixit, 1995), suggesting ICE involvement in these signaling pathways as well. Therefore, it is not surprising that TRADD-mediated cell death involves ICE, or an ICE-related protease; this result might be expected if TRADD overexpression were to activate a latent TNFR1 apoptotic pathway.

The Fas antigen signals cell death through a portion of its cytoplasmic domain that is 28% identical to the death domain of TNFR1 (Itoh and Nagata, 1993). Interestingly, TRADD does not appear to interact with Fas antigen. This observation suggests that the receptor-proximal apoptotic signaling pathways activated by TNFR1 and Fas antigen are distinct, but converge downstream at some point before ICE activation. Alternatively, the two pathways may be parallel and activate distinct crmA-inhibitable, ICE-related proteases. In either case, it is possible that a TRADD-related protein will be discovered that associates with the death domain of Fas antigen. Furthermore, both alternatives are consistent with reports that TNF and Fas activate programs of cell death that are at least partially distinct (Wong and Goeddel, 1994; Schulze-Osthoff et al., 1994).

Both TNF and IL-1 induce the transcriptional activation of a large set of genes involved in acute phase and inflammatory responses by activating the transcription factor NF-κB. In general, the proinflammatory activity of TNF is signaled through TNFR1 (Kruppa et al., 1992; Pfeffer et al., 1993), although there are some cell lines in which TNFR2 can activate NF-κB (Rothe et al., 1994; Lægreid et al., 1994). Transient overexpression of TRADD potently activated NF-κB in several TNF-responsive cell lines. However, in several instances this activation could be seen only when the cell death pathway was inhibited by crmA expression. Our failure to observe any interaction between TRADD and either IL-1R1 or TNFR2 suggests that TRADD is not involved in NF-κB activation initiated by these two receptors and may be exclusively dedicated to TNFR1 signal transduction.

Deletion mutagenesis experiments identified a region of 111 amino acids near the C-terminus of TRADD that is sufficient to trigger both apoptosis and NF-κB activation. This death domain is also sufficient for interaction with TNFR1 and self-association. Furthermore, this region is related in primary sequence to the death domain of TNFR1, but bears little similarity to the Fas antigen death domain. This raises the possibility that the greater se-

qu nce identity betw n TRADD and TNFR1 d fin s a structural framework that accounts for th ir ability to interact specifically.

To date, we have been unable to identify a TRADD mutant that interacts with TNFR1 but fails to signal. Such a mutant might be expected to act as a dominant negative inhibitor of TNF signaling, thereby demonstrating an essential role for TRADD in TNFR1 signal transduction. However, strong correlative evidence for such a role comes from several observations. First, no active TRADD deletion mutants were found that failed to interact with TNFR1. Second, mutants of TNFR1 that are incapable of signaling cell death or NF-κB activation do not interact with TRADD. Third, two-hybrid screening using murine TNFR1 as bait resulted in the isolation of a cDNA for murine TRADD (M. Pan and D. V. G., unpublished data). Finally, human TRADD is able to interact with the cytoplasmic domains of murine and human TNFR1, which do not exhibit species specificity, yet share only 59% sequence identity (Lewis et al., 1991).

Mechanisms of TRADD-Induced Signal Transduction

The disparate effects of crmA expression on TRADD-induced apoptosis and NF-κB activation are significant, as they suggest divergence of the apoptotic and NF-κB pathways mediated by TNFR1. Furthermore, the failure of crmA expression to block NF-κB activation indicates that this aspect of TRADD activity is not an artifact of sick or dying cells. Apparently, the interaction of the death domain of TNFR1 with the C-terminal region of TRADD activates either two separate signaling cascades or one primary signal that subsequently splits into two (or more) pathways before ICE is encountered on the apoptotic pathway.

Just how TRADD is able to transmit signals downstream from TNFR1 remains a mystery at this time. Western blot analyses indicate that TRADD is normally expressed in very low amounts. On the basis of cDNA cloning results (approximately one positive in 10^6 clones screened) and Northern blot analyses, we estimate that *TRADD* mRNA levels are typically less than 5 molecules per cell. Perhaps TRADD has a higher affinity for aggregated than for monomeric TNFR1, such that under normal physiological conditions, interaction can only occur following TNF-induced receptor aggregation. Another possibility is that a TRADD or TNFR1-associated inhibitory protein prevents interaction of TRADD with TNFR1 prior to receptor aggregation. Both possibilities might be expected to represent delicately balanced signaling systems that could be overridden by enforced expression of TRADD. Similarly, overexpression of TNFR1 has be n shown to trigg r c ll death, IL-8 gene induction (Boldin et al., 1995), and NF-κB activation (H. H. and D. V. G., unpublished data). In this case, ligand-independ nt aggregation of TNFR1 is observed, which might result in r cruitment of TRADD to the receptor complex.

The identification and characterization of TRADD described herein provides an initial st p in deciph ring the intracellular signaling pathways activated by TNF binding to TNFR1. Howev r, many important questions remain unr solved: first, what role does TRADD play in oth r TNFR1-transduced signals, such as activation of sphingomyelinases or the TNFR1-associated serine kinase activity? Second, what is the function of the ~200 N-terminal amino acids of TRADD that are not required for induction of apoptosis and NF-κB activation? Third, how does TRADD connect to downstream signaling events? Fourth, do other members of the TNF receptor superfamily interact with TRADD or related molecules to initiate their signaling cascades? Finally, do TRADD-like proteins participate in other (non-TNF receptor superfamily–mediated) pathways of programmed cell death? We hope that the molecular reagents and observations reported herein will help stimulate a resolution to these and other questions.

Experimental Procedures

Reagents and Cell Lines

Recombinant human TNF was provided by Genentech, Incorporated. The rabbit anti-TNFR1 polyclonal antibody was described previously (Tartaglia et al., 1991). The rabbit anti-TRADD antiserum was raised against a GST–TRADD fusion protein by BabCo (Richmond, California). The monoclonal antibody against the Myc epitope (S-M-E-Q-K-L-I-S-E-E-D-L-N) was provided by R. Schreiber. Rabbit anti-p50 and anti-p65 polyclonal antibodies were purchased from Santa Cruz Biotechnology. The HtTA-1 (for HeLa expressing a tetracycline-controlled transactivator; Dr. H. Bujard), 293 (R. Tjian), HepG2 (American Type Culture Collection [ATCC]), ECV304 (ATCC), and NIH 3T3 (S. McKnight) cell lines were obtained from the indicated sources.

Expression Vectors

The *TRADD* cDNA was cloned as a 1.4 kb EcoRI fragment into pRK5 under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter–enhancer (Schall et al., 1990). The resulting plasmid, pRK-TRADD, was used for mammalian cell expression and for in vitro transcription and translation using the SP6 promoter. Myc epitope tag constucts were made by replacing the eight N-terminal codons of *TRADD* with DNA encoding the sequence M-A-S-M-E-Q-K-L-I-S-E-E-D-L. C-terminal deletion mutants of TRADD were generated by replacement of sequences between the XhoI site in *TRADD* and the HindIII site in pRK5 with synthetic DNA containing the appropriate coding sequence and in-frame stop codons. N-terminal deletion mutants of TRADD were generated by polymerase chain reaction (PCR). The various mutant *TNFR1* expression vectors were described previously (Tartaglia et al., 1993). A plasmid containing the cowpox virus *crmA* gene (Pickup et al., 1986) was obtained from G. Palumbo. A 1.0 kb *crmA* fragment was generated by PCR and inserted into the pRK5 vector to give the plasmid pRK–crmA. The *Bcl-2* expression vector pSFFV–Bcl-2, based on the long terminal repeat of the splenic focus-forming virus, was provided by S. Korsmeyer. The CMV-based expression vector pCMV19K for the adenovirus 19 kDa E1B protein was provided by J. Fraser. The NF-κB–luciferase reporter plasmid pELAM-luc, containing E-selectin promoter sequences from position −730 to position +52 (Schindler and Baichwal, 1994), was provided by U. Schindler.

Yeast Two-Hybrid Cloning

DNA encoding the intracellular domain (amino acids 214–426) of TNFR1 was cloned into the yeast GAL4 DNA-binding domain vector pGBT9. The resulting plasmid, pGAL4bd–TNF–R1icd, was used as bait in two-hybrid screens of HeLa and B cell cDNA libraries (Clontech) following the Matchmaker Two-Hybrid System Protocol (Clontech). Positive yeast clones were selected by prototrophy for histidine and expression of β-galactosidase. Yeast DNA was recovered and transformed into E. coli. Plasmids containing cDNA clones were identified by restriction mapping and further characterized by DNA sequencing. Subsequent two-hybrid interaction analyses were carried out by co-transformation of plasmids containing the GAL4 DNA-binding (pGBT9)

and -activation (pGAD424) domains into Saccharomyces cerevisiae strain SFY526.

cDNA Cloning and Northern Blot Hybridization

The cDNA insert of approximately 1 kb from two-hybrid clone B27 was used as probe to screen human HeLa and HUVEC cDNA libraries in λgt11 (provided by Dr. Z. Cao; complexity of >2 × 10^6 clones each) by standard methods (Sambrook et al., 1989). Two independent positive clones were obtained from each library. Following subcloning into pBluescript KS (Stratagene), DNA sequencing was performed on an Applied Biosystems, Incorporated model 373A automated DNA sequencer by use of the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems). Northern blot analysis of the human multiple tissue blot (Clontech) was performed according to the instructions of the manufacturer, by use of an ~400 bp EcoRI–NarI fragment from the 5′ end of *TRADD* cDNA as probe.

Cell Culture, Transfections, and Reporter Assays

293 and NIH 3T3 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin (GIBCO). HtTA-1 (HeLa) cells were grown in the same medium containing 400 μg/ml G418. HepG2 cells were maintained in DMEM/F12 (1:1) medium with the same additives. For reporter assays, coimmunoprecipitations, and cell killing assays, ~2 × 10^5 cells/well were seeded on 6-well (35 mm) dishes and grown in 5% CO_2 at 37°C. Cells were transfected the next day by the calcium phosphate precipitation method (Ausubel et al., 1994). After an incubation of 24–36 hr, cells were washed twice with phosphate-buffered saline and then lysed with 200 μl of lysis buffer (25 mM Tris–phosphate [pH 7.8], 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid [CDTA], 10% glycerol, 1% Triton X-100). Aliquots of cell lysates [20 μl] were mixed with 100 μl of luciferase assay reagent (Promega) and the luciferase activity determined using a Model 20e luminometer (Turner Designs). β-Galactosidase activity was determined in a mixture containing 10 μl of cell lysate, 10 μl of 50 mM chlorophenol red β-D-galactopyranoside (CPRG), and 80 μl of Z buffer (60 mM Na_2HPO_4, 10 mM KCl, 1 mM β-mercaptoethanol [pH 7.0]). Samples were incubated at 37°C until red color developed, and absorbance was determined at 574 nm. These values were used to normalize transfection efficiencies. For DNA laddering and EMSA experiments, transfections were performed with 100 mm dishes seeded with ~10^6 cells. At 24–36 hr after transfection, DNA or nuclear extracts were prepared.

Coimmunoprecipitations and Western Blot Analysis

Western blot analysis to detect TRADD was performed with the anti-TRADD antiserum and horseradish peroxidase–coupled goat anti-rabbit IgG (Amersham) using enhanced chemiluminescence according to the protocol of the manufacturer. For immunoprecipitation assays, 50 μl aliquots of lysates from transfected cells were incubated with 1 μl of the anti-TNFR1 antibody and 450 μl of E1A buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA). The mixture was incubated at 4°C for 1 hr, then mixed with 20 μl of a 1:1 slurry of protein A–Sepharose (Pharmacia) and incubated for another hour. The beads were washed twice with 1 ml of E1A buffer, twice with 1 ml of high salt (1 M NaCl) E1A buffer, and twice again with E1A buffer. The precipitates were fractionated on 10% SDS–polyacrylamide gels and transferred to Immobilon P membrane (Millipore). The blot was subjected to Western blot analysis with anti-Myc monoclonal antibody and horseradish peroxidase–coupled rabbit anti-mouse immunoglobulin.

Generation of GST Fusion Proteins and In Vitro Binding Assays

TRADD and the cytoplasmic regions of TNFR1, TNFR1(−20), Fas antigen, IL-1R1, and TNFR2 were expressed individually as GST (glutathione S-transferase) fusion proteins by use of pGEX vectors (Pharmacia). Expression and purification of the GST fusion proteins were performed as described (Smith and Johnson, 1988). ^{35}S-labeled proteins were generated with the TNT SP6 Coupled Reticulocyte Lysate System (Promega) and the various *TRADD* expression constructs in pRK5. For each in vitro binding assay, 10 μl of glutathione–Sepharose beads (Pharmacia) bound to the appropriate GST fusion protein (~5 μg) was incubated with ^{35}S-labeled polypeptides in 1 ml of E1A buffer at 4°C for 1 hr. The beads were then washed six times with E1A buffer. Proteins on the beads were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and exposed to Kodak X-ray film.

Apoptosis Assays

DNA fragmentation assays were performed as described (Hermann et al., 1994), using approximately 10^7 transfected or nontransfected cells. DNA aliquots (150 ng) were fractionated by electrophoresis in a 1.6% agarose gel.

β-Galactosidase cotransfection assays for determination of cell death were performed as described by Kumar et al. (1994). Cells were observed microscopically, and the number of blue cells per 35 mm well was determined by counting.

EMSAs

Nuclear extracts were prepared as described by Osborn et al. (1989). Double-stranded oligonucleotides (5′-GATGCCATTGGGGATTTCCT-CTTTACTG) containing an NF-κB-binding site were ^{32}P-labeled using polynucleotide kinase. Each gel shift assay was performed in a 30 μl reaction mixture containing 10 μg of nuclear extracts, 0.4 ng of radiolabeled oligonucleotide probe, 1 μg of sonicated E. coli DNA, 6 μl of 5× EMSA buffer (100 mM HEPES [pH 7.6], 250 mM KCl, 5 mM DTT, 5 mM EDTA, 25% glycerol), and where necessary, 20 μg of cold competitor oligonucleotides. The mutant competitor oligonucleotides have the underlined NF-κB site changed to GGaagcTTCC. Mixtures were incubated at room temperature for 10 min and then subjected to electrophoretic fractionation on a 5% polyacrylamide gel at 4°C.

Acknowledgments

We thank Keith Williamson for performing DNA sequencing, Dean Stott for oligonucleotide synthesis, Zhaodan Cao for providing cDNA libraries, and Glenn Croston and Mike Rothe for GST–IL-1R1 and GST–TNFR2, respectively. We also thank Mike Rothe and Steve McKnight for discussions and suggestions on the manuscript, and Laura Medin for help in preparing the manuscript and figures.

Received March 8, 1995; revised March 24, 1995.

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GenBank Accession Number

The accession number for the human TRADD sequence reported in this paper is L41690.

Cell, Vol. 81, 505–512, May 19, 1995

FADD, a Novel Death Domain-Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis

Arul M. Chinnaiyan,* Karen O'Rourke,* Muneesh Tewari, and Vishva M. Dixit
Department of Pathology
University of Michigan Medical School
Ann Arbor, Michigan 48109

Summary

Using the cytoplasmic domain of Fas in the yeast two-hybrid system, we have identified a novel interacting protein, FADD, which binds Fas and Fas-FD5, a mutant of Fas possessing enhanced killing activity, but not the functionally inactive mutants Fas-LPR and Fas-FD8. FADD contains a death domain homologous to the death domains of Fas and TNFR-1. A point mutation in FADD, analogous to the *lpr* mutation of Fas, abolishes its ability to bind Fas, suggesting a death domain to death domain interaction. Overexpression of FADD in MCF7 and BJAB cells induces apoptosis, which, like Fas-induced apoptosis, is blocked by CrmA, a specific inhibitor of the interleukin-1β-converting enzyme. These findings suggest that FADD may play an important role in the proximal signal transduction of Fas.

Introduction

Programmed cell death (PCD) is a physiological process essential to the normal development and homeostatic maintenance of multicellular organisms (reviewed by Vaux et al., 1994; Ellis et al., 1991). Apoptosis, often equated with PCD, refers to the morphological alterations exhibited by "actively" dying cells that include cell shrinkage, membrane blebbing, and chromatin condensation (Cohen, 1993). In contrast, necrosis, sometimes referred to as accidental cell death, is defined by the swelling and lysis of cells that are exposed to toxic stimuli.

Though the morphological features of cell death are well described, the molecular mechanisms behind apoptosis remain undefined. Recent work on PCD in the nematode Caenorhabditis elegans showed that the *ced-3* gene is required for apoptosis (Yuan et al., 1993). Sequence analysis revealed that CED-3 is similar to the mammalian interleukin-1β (IL-1β)-converting enzyme (ICE) (Yuan et al., 1993), which is a cysteine proteinase involved in the processing and activation of pro-IL-1β to the active cytokine (Cerretti et al., 1992; Thornberry et al., 1992). Overexpression of ICE in mammalian cells induced apoptosis, suggesting that ICE, or a related protease, may be an essential component of the cell death pathway (Miura et al., 1993).

Although a CED-3-like protease is suspected to be a distal effector of the mammalian cell death pathway, the proximal components that lead to its activation remain to be identified. Two cell surface cytokine receptors, Fas/APO-1 antigen and the receptor for tumor necrosis factor (TNF), have been shown to trigger apoptosis by natural ligands or specific agonist antibodies (Baglioni, 1992; Yonehara et al., 1989; Itoh et al., 1991; Trauth et al., 1989). Mice carrying a point mutation in the cytoplasmic domain of Fas exhibit a lupus-like lymphoproliferative autoimmune disorder (*lpr*), (Watanabe-Fukunaga et al., 1992), and the Fas-mediated cell death pathway has been recently implicated in the activation-induced death of T cells (Dhein et al., 1995; Brunner et al., 1995; Ju et al., 1995). While the main activity of Fas is to trigger cell death, the TNF receptor (TNFR) can signal an array of diverse activities such as fibroblast proliferation, resistance to chlamidiae, and synthesis of prostaglandin E2 (Tartaglia and Goeddel, 1992). Recent work in our laboratory suggests that the stimulation of Fas or the TNFR triggers the activation of a common component of the cell death pathway. CrmA, a poxvirus gene product, was shown to potently block both Fas- and TNF-induced cell death (Tewari and Dixit, 1995). Interestingly, the only reported target for CrmA is the CED-3 homolog ICE (Ray et al., 1992), suggesting that ICE, or an ICE-like protease, is the common effector of cytokine receptor–mediated cell death.

The activation of Fas and TNFR is caused by receptor aggregation mediated by the respective ligands or agonist antibodies. The signal is thought to be transduced by clustering of the intracellular domain (Boldin et al., 1995; Song et al., 1994), which encompasses a region that is significantly conserved in the Fas antigen as well as in TNFR-1 (Tartaglia et al., 1993; Itoh and Nagata, 1993). This shared "death domain" suggests that both receptors interact with a related set of signal transduction molecules that, thus far, remain unidentified.

Here, we report the molecular cloning and characterization of FADD, a Fas-associating protein with a novel death domain. The specific interaction of Fas and FADD is due to the association of their respective homologous death domains. Remarkably similar to Fas-induced killing, overexpression of FADD induces apoptosis that is inhibitable by CrmA. Taken together, our results suggest that FADD is a component of the Fas-mediated cell death pathway.

Results

Isolation of FADD

The yeast two-hybrid system was used to screen for proteins that interact with the cytoplasmic domain of Fas. An expression vector was constructed by fusing the GAL4 DNA-binding domain to the cytoplasmic tail of the human Fas antigen (GAL4bd–Fas). This bait plasmid was cotransformed in yeast with a prey plasmid containing a human B cell cDNA expression library fused to the GAL4 activation domain. Seventeen positive clones were obtained from 2×10^6 transformants screened. To determine the specificity of interaction, plasmids containing the activation domain fusion proteins were recovered from the putative positive clones and cotransformed with GAL4bd–Fas and control heterologous baits. Two clones (8 and 15) were

*The first two authors contributed equally to this work

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AU   Stanger B Z; Leder P; Lee T H; Kim E; Seed B
TI   RIP: a novel protein containing a death domain that interacts with
     Fas/APO-1 (CD95) in yeast and causes cell death.
SO   CELL, (1995 May 19) 81 (4) 513-23.
     Journal code: CQ4. ISSN: 0092-8674.
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RIP: A Novel Protein Containing a Death Domain That Interacts with Fas/APO-1 (CD95) in Yeast and Causes Cell Death

Ben Z. Stanger,*† Philip Leder,*† Tae-Ho Lee,†‡ Emily Kim,†‡ and Brian Seed†‡
*Howard Hughes Medical Institute
†Department of Genetics
Harvard Medical School
Boston, Massachusetts 02115
‡Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts 02114

Summary

Ligation of the extracellular domain of the cell surface receptor Fas/APO-1 (CD95) elicits a characteristic programmed death response in susceptible cells. Using a genetic selection based on protein–protein interaction in yeast, we have identified two gene products that associate with the intracellular domain of Fas: Fas itself, and a novel 74 kDa protein we have named RIP, for receptor interacting protein. RIP also interacts weakly with the p55 tumor necrosis factor receptor (TNFR1) intracellular domain, but not with a mutant version of Fas corresponding to the murine *lpr*^{cg} mutation. RIP contains an N-terminal region with homology to protein kinases and a C-terminal region containing a cytoplasmic motif (death domain) present in the Fas and TNFR1 intracellular domains. Transient overexpression of RIP causes transfected cells to undergo the morphological changes characteristic of apoptosis. Taken together, these properties indicate that RIP is a novel form of apoptosis-inducing protein.

Introduction

Although regulated cell death is known to be essential for the orderly development of metazoan organisms and is crucial to the proper functioning of the immune system in higher vertebrates, relatively little is known about the mechanisms by which cell death programs are executed. One important mediator of immunologically relevant cell death processes is the Fas antigen/APO-1 (CD95), originally identified as the target of monoclonal antibodies that could kill multiple cell types (Trauth et al., 1989; Yonehara et al., 1989). cDNA cloning and sequence analysis (Itoh et al., 1991; Watanabe-Fukunaga et al., 1992a; Oehm et al., 1992) showed Fas to be a member of a family of transmembrane receptors that includes the low affinity nerve growth factor (NGF) receptor, the tumor necrosis factor receptors (TNFR1 and TNFR2), and a variety of immune cell receptors including CD40, OX40, CD30, CD27, and 4-1BB (for review see Smith et al., 1994). Several members of this family besides Fas have been shown to regulate or induce cell death, in particular the p55 TNFR (TNFR1) (Tartaglia et al., 1991, 1993b) and the p75 TNFR (TNFR2) (Heller et al., 1992, 1993; Clement and Stamenkovic, 1994).

Disruption of Fas expression or function in lymphoproliferation (*lpr*) mutant mice leads to a progressive lymphadenopathy and autoimmune syndrome resembling human systemic lupus erythematosus (Watanabe-Fukunaga et al., 1992b). The residual cytotoxic activity of T cells derived from perforin-deficient mice is also dependent on the presence of at least one wild-type allele of the *lpr* locus (Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994). Thus, the ability of Fas to induce cell death is important for the maintenance of at least two immunological processes in vivo: peripheral tolerance to self (Singer and Abbas, 1994; Crispe, 1994) and calcium-independent T cell cytotoxicity (reviewed by Henkart, 1994).

The mechanism by which Fas induces cell death is unknown, but it requires multivalent cross-linking of the receptor (Dhein et al., 1992) and is facilitated by concurrent inhibition of RNA or protein synthesis in some cell types. Other factors have been reported to modulate Fas activity (Klas et al., 1993), and under certain circumstances, Fas is capable of signaling activation rather than death (Alderson et al., 1993). Anti-Fas antibodies and TNF are both capable of signaling cell death in vitro with similar kinetics (Yonehara et al., 1989; Itoh and Nagata, 1993), and among members of the NGF/TNF receptor family, Fas and TNFR1 share the most significant cytoplasmic homology.

Deletion and mutational analyses have led to the identification of sequences within Fas that are required for the cell death response (Itoh and Nagata, 1993). It has been proposed that a death domain contained in a region of similarity between Fas and TNFR1 is essential for the initiation of apoptosis by both molecules, perhaps through an interaction with other intracellular proteins (Tartaglia et al., 1993a).

To study the events elicited by Fas ligation, we have exploited a yeast protein interaction system to identify a novel protein that interacts with the Fas cytoplasmic domain. This protein, which we have named RIP (for receptor interacting protein), contains a death domain homology region at its C-terminus and a kinase domain at its N-terminus. Overexpression of RIP leads to morphological changes characteristic of apoptosis. The ability of RIP to associate with the Fas intracellular domain in yeast and to promote apoptotic changes upon overexpression in mammalian cells suggests it may be an important element in the signal transduction machinery that mediates programmed cell death.

Results

Identification of cDNAs Encoding Proteins Domains That Bind Fas

A cDNA library screen for proteins that interact with the intracellular domain of Fas was conducted with the help of a yeast genetic selection system (Gyuris et al., 1993; Zervos et al., 1993). A cDNA segment comprising virtually the entire cytoplasmic domain of human Fas (residues

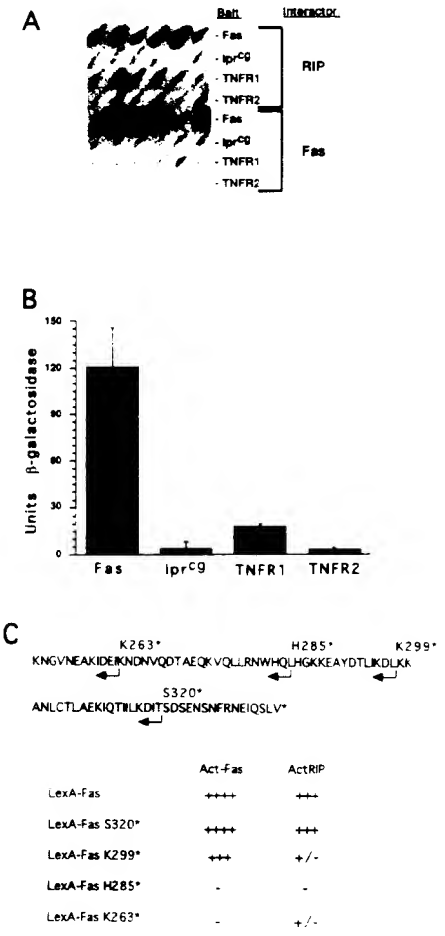

Figure 1. Specificity of RIP Interactions in Yeast

(A) RIP–Fas and Fas–Fas interactions. Cells of the S. cerevisiae strain EGY48/pRB1840 were sequentially transformed with the indicated LexA–fusion plasmid and either the Act–Fas or the Act–RIP library isolate. Independent colonies arising from the activator plasmid transformations were streaked on Ura⁻ His⁻ Trp⁻ plates containing X-Gal, galactose, and raffinose and photographed using a red filter.

(B) Quantitative β-gal assay. Three colonies from each Act–RIP/LexA–fusion protein pair were used to inoculate a galactose-containing liquid culture. The β-gal activity of lysates prepared from each culture was measured and normalized to the total protein concentration of the lysate. 5 units (nmoles/min/mg protein) represents the limit of detection of β-gal activity in this system.

(C) Pairwise association between LexA–Fas deletion variants and Act–Fas or Act–RIP. The indicated yeast strains were constructed by transformation, and the production of β-gal was tested by a chromogenic colony assay using X-Gal. The extent of color development of individual colony streaks was scored visually, with ++++ indicating dark blue and - indicating the growth of white colonies only. +/- indicates the presence of faint blue flecks in some of the colonies bearing the indicated Act/LexA pair.

192–329 of the Fas precursor) was fused to the 3′ end of the coding region for the bacterial repressor LexA, and a yeast expression plasmid containing this gene fusion was introduced into a reporter strain harboring the Saccharomyces cerevisiae LEU2 and Escherichia coli β-galactosidase (β-gal) coding sequences under the control of a synthetic promoter bearing LexA-binding sites. The resulting yeast strain was transformed with a transcriptional activator fusion protein library prepared from mRNA isolated from the Jurkat (human T cell leukemia) cell line, which is known to undergo apoptosis when subjected to treatment with anti-Fas antibody. Transformants were plated on selective (Leu-deficient) plates containing galactose, which induces the *GAL1* promoter that directs transcription of the library insert. Leu prototrophs were transferred to plates containing X-Gal and galactose, and colonies giving a dark blue color were recovered and analyzed further.

To test the specificity of the interaction between the candidate interaction partners and Fas, the library plasmids were reintroduced into a second strain harboring a LexA–Fas fusion gene (LexA–lpr^{cg}) in which the Fas portion had been mutated by substitution of Asn for Val at position 254 of the Fas precursor sequence. This mutation was expected to exhibit a molecular phenotype similar to that of the murine *lpr^{cg}* allele, which is formed by substitution of Asn for Ile at the homologous position. None of the candidate plasmids showing evidence of strong interaction with LexA–Fas were capable of interacting with LexA–lpr^{cg} (Figure 1A; data not shown).

Restriction site and sequence analysis of the cDNA inserts of the candidate clones showed that they fell into only two classes, each of which consisted of an incomplete cDNA. One of the inserts encoded the C-terminal residues 222–335 of the intracellular domain of Fas itself, and the other encoded a protein, subsequently named RIP, without overt relationship to previously described polypeptides. For simplicity in the following discussion, we will refer to the transcriptional activator fusion proteins expressed by the two classes of library isolates as Act–Fas and Act–RIP.

The specificity of the interaction partners was further tested by using the library plasmids to transform yeast harboring expression plasmids encoding LexA fusions with intracellular domains of various cell surface receptors (Figure 1A). Although no interaction was detected in most cases (data not shown), weak promoter activity was discerned following introduction of the yeast plasmid encoding Act–RIP into strains harboring LexA–TNFR1 intracellular domain. By contrast, no activity was seen when Act–RIP was introduced into strains harboring LexA–TNFR2 intracellular domain. Yeast transformed with Act–Fas displayed promoter activity in strains harboring LexA–Fas intracellular domain, but showed no activity in strains harboring any other LexA–intracellular domain (Figure 1A).

To measure this effect more precisely, β-gal assays were performed on lysates of yeast harboring various pairs of LexA–intracellular domain and Act–RIP. Lysates from yeast bearing LexA–Fas and Act–RIP contained about 30- to 40-fold more β-gal activity than strains bearing Act–RIP and either LexA–lpr^{cg} or LexA–TNFR2 (Figure 1B). Lysates prepared from yeast harboring LexA–TNFR1 and Act–RIP expressed β-gal activity at about 10% of the level seen in lysates prepared from yeast bearing LexA–Fas and Act–RIP (Figure 1B).

One explanation for the failure to detect an interaction between Act–RIP and LexA–lpr^{cg} or LexA–TNFR2 could have been that the LexA fusion proteins were poorly ex-

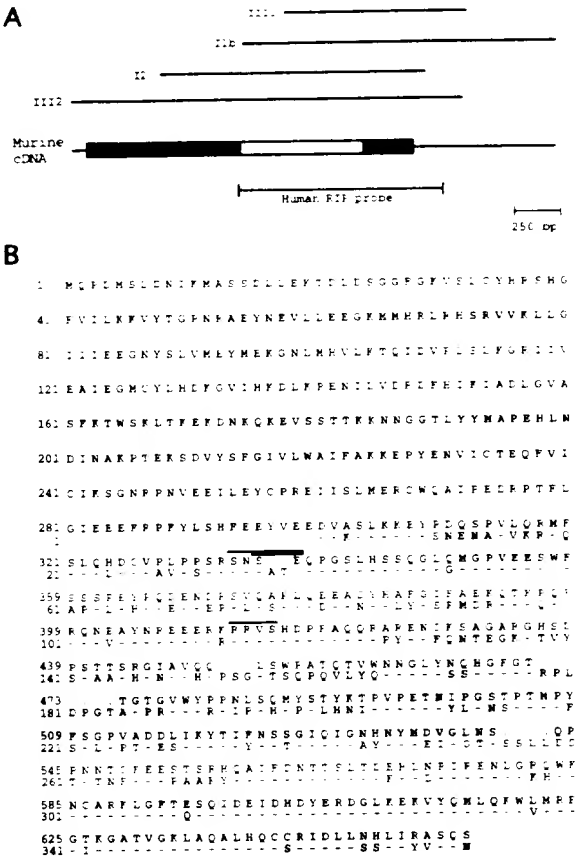

Figure 2. Structure of RIP cDNAs and Deduced Amino Acid Sequence

(A) Schematic diagram of RIP cDNA. The inserts of clones isolated from a mouse thymus cDNA library are symbolized as lines above a bar diagram of the composite sequence, which depicts the regions encoding the kinase domain (stippled box), the death domain (closed box), and a region of unknown function predicted to have high α-helical content (open box). Also shown is the region of human RIP that was used as a probe to isolate murine cDNAs. The original human RIP isolate encompassed sequences encoding the death domain and about 100 residues upstream.

(B) Inferred polypeptide sequence. The mouse sequence consists of a contiguous open reading frame proceeding from a translational initiation consensus. The human sequence predicted from a cDNA fragment is shown below the mouse; identical residues are indicated by a dash, and gaps indicated by a period. The conserved consensus sequences for casein kinase II (S–X–X–E) and cAMP- or cGMP-dependent protein kinase (R–X–X–S) are overlined (reviewed by Kennelly and Krebs, 1991).

pressed. To address this possibility, a portion of each lysate used to measure enzyme activity was subjected to gel electrophoresis and blot transfer, followed by detection with anti-LexA antiserum. LexA fusion proteins of the appropriate size were detected in each of the lysates, and both the LexA–Ipr^{cg} and LexA–TNFR2 fusion proteins were found to be more abundantly expressed than LexA–Fas or LexA–TNFR1 (data not shown), making it unlikely that failure to detect interaction in vivo could be attributed to degradation or inadequate synthesis of the LexA chimeras.

To localize the sequences in Fas/APO-1 that are responsible either for self-interaction or for interaction with RIP, we prepared a set of LexA–Fas C-terminal deletion chimeras and tested their ability to support interaction with either Act–Fas or Act–RIP, as measured by β-gal colony assay (Figure 1C). These studies showed that the 16 C-terminal residues of Fas are not required for interaction with either RIP or Fas itself, but that removal of additional residues severely compromises the association with RIP.

Cloning and Structure of Murine RIP

Because the RIP cDNA insert identified by genetic selection in yeast did not encode an open reading frame bearing a consensus translational initiation sequence, additional human cDNA libraries were screened by hybridization for a full-length clone. None were identified, however, and several of the resulting isolates appeared to terminate at approximately the same 5′ terminus, suggesting that secondary structure in the mRNA might have prevented extension of the cDNA by reverse transcriptase. Although the largest clone spanned approximately 1 kb of sequence, preliminary RNA blot hybridizations revealed a transcript of approximately 4.2 kb expressed in cell lines of diverse provenance, including tumors of lymphoid, hepatic, renal, neuronal, cervical, intestinal, muscular, and skeletal origin.

To isolate a full-length clone, the human RIP coding sequence was used to probe a mouse thymus cDNA library. Four distinct overlapping clones were identified, ranging in size from 1 kb to 2.4 kb, as shown in Figure 2A. Restriction analysis and sequencing revealed that one of these clones, III2, extended further 5′ than the others. The 2.3 kb insert of III2 contains a 1968 nt open reading frame beginning with a translational initiation consensus sequence (Kozak, 1987) and predicting a polypeptide of 656 amino acids with a M_r of 74,000 (Figure 2B).

The N-terminal region of RIP bears an extended homology to protein kinases that begins a few residues after the presumptive initiating Met and extends to the vicinity of residue 300. Quantitative sequence comparisons based on a word match algorithm (Altschul et al., 1990) predict that this domain is most similar overall to the Tyr subclass of protein kinases, with the highest relatedness seen to the mouse *lck* gene product (Marth et al., 1985). However, in the key subdomains that discriminate most closely between Tyr and Ser/Thr substrate specificity, the DLKPEN sequence (corresponding to kinase subdomain VI) and the GTLYYMAPE sequence (kinase subdomain VIII), RIP appears to match the Ser/Thr family consensus (Figure 3A; Hanks and Lindberg, 1991; Taylor and Radzio-Andzelm, 1994). Residues that are conserved in kinases of both subclasses (for example, kinase subdomain IX, Figure 3A) are also well conserved in RIP.

The sequence predicted by the longest of the human cDNA fragments consists of 375 amino acids corresponding to the region just C-terminal to the kinase domain of murine RIP and shares 67% sequence identity with the murine sequence over this length. Within this domain, the first 270 amino acids following the kinase domain have no striking homology to other proteins, although a small subdomain is highly conserved between mouse and human proteins (residues 391–427 of the murine sequence) and has a relatively high representation of Arg (R), Gln

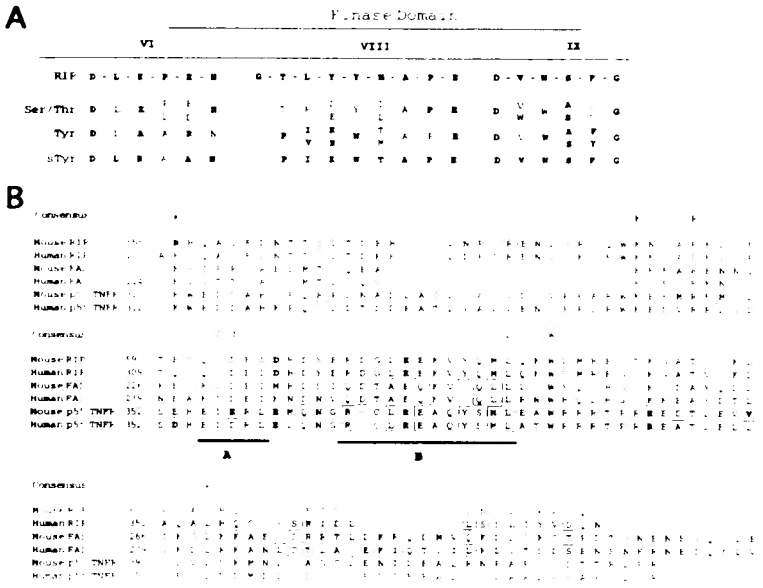

Figure 3. RIP is Similar to Proteins with Kinase and Death Domains

(A) Comparison of RIP to the catalytic domains of Ser/Thr and Tyr kinases and the Src subfamily of Tyr kinases (sTyr). Consensus sequences were described by Hanks and Lindberg (1991) and represent analysis of 70 Ser/Thr kinases, 26 Tyr kinases, and 8 sTyr kinases. Consensus residues found in over 95% of sequences analyzed are bolded.

(B) Sequence alignment of the RIP C-terminus with the death domains and C-termini of Fas and TNFR1. Gaps are indicated by dashes. Consensus residues conserved in all six sequences are capitalized, whereas positions at which a charge-conserved residue is found in one of the sequences are shown in lower case. Noteworthy charge conservations are bolded. The regions denoted (A) and (B) represent two portions of the Fas cytoplasmic domain completely conserved between mouse and human.

(Q), and Glu (E) (18/37 residues in both sequences). The sequence of this region is similar to portions of the trichohyalin family of hair structural proteins, which contain RQE-rich repeats that form highly stabilized α helices (Lee et al., 1993).

The RIP C-Terminus Has Death Domain Homology

The 98 C-terminal amino acids of RIP share 87% sequence identity between mouse and human, suggesting they subserve some regulatory function (Figure 2B). Comparable domains of approximately 90 residues close to the C-termini of Fas and TNFR1 have been shown to play a role in the transduction of apoptotic signals to receptive cells and have been termed death domains for this reason (Tartaglia et al., 1993a). Pairwise comparisons of the death domain sequences aligned in Figure 3B showed the highest relatedness between human RIP and human TNFR1 (59% similarity and 30% identity), which are significantly more similar than human Fas and human TNFR1 (42% similarity and 23% identity). The interspecies conservation of the RIP death domain (84% identity between mouse and human) exceeds that of the TNFR1 (68% identity) and Fas (49% identity) death domains.

Constitutive and Inducible Expression of RIP mRNA

Preliminary RNA blot hybridization experiments demonstrated the existence of an RNA species of approximately 3.8 kb in a variety of cell lines. To more precisely assess mRNA abundance in tissues, a quantitative ribonuclease protection assay was employed. Use of a labeled antisense RNA probe corresponding to the 3′ terminus of the cDNA gave rise to a ribonuclease-resistant species of the expected size in all adult tissues tested (Figure 4A). An in vitro labeled RNA antisense to the mRNA for the ribosomal large subunit protein L32 was used as an internal standard to allow normalization to the amount of RNA loaded in each lane. Analysis of the protected RNA showed that RIP mRNA levels varied by less than 2- to 3-fold between most tissues (data not shown); lung showed the highest expression, whereas tongue showed the least.

The possibility that RIP mRNA might be regulated as a consequence of activation in T cells was also explored. Dissociated murine splenocytes were stimulated in vitro with the lectin–concanavalin A (ConA), and total RNA prepared at various times following addition of lectin was analyzed for the presence of RIP sequences by RNA blot analysis. Little or no RIP RNA could be detected in unstimulated splenocytes, but a single 3.8 kb species appeared in unfractionated splenocytes that had been exposed to lectin for 2 hr or longer (Figure 4B). Since RIP mRNA is detectable by ribonuclease protection in the spleen as a whole (Figure 4A), the inability to detect RIP mRNA in splenocytes treated with ConA for less than 2 hr is probably due to the lower sensitivity of RNA blot analysis, although this discrepancy could also have resulted from RIP expression exclusively in the fibrous tissue of the spleen.

A Polypeptide of the Expected Molecular Mass Is Expressed In Vivo

To examine the distribution of RIP protein in vivo, a rabbit antiserum was prepared against a fusion protein consisting of the 250 C-terminal residues of murine RIP fused to E. coli maltose-binding protein. The antiserum specificity was validated by immunoprecipitation of RIP synthesized in vitro. Following in vitro transcription and translation of the RIP open reading frame, a single labeled product of approximately 74 kDa was observed that could be specifically immunoprecipitated with the rabbit antiserum, but not with serum from unimmunized animals (Figures 5A and 5B). The specificity of the antiserum for RIP was also documented by its inability to immunoprecipitate an irrelevant protein (Photinus pyralis luciferase) similarly translated in vitro. Immunoprecipitation of a lysate of metabolically labeled NIH 3T3 cells with the rabbit antiserum

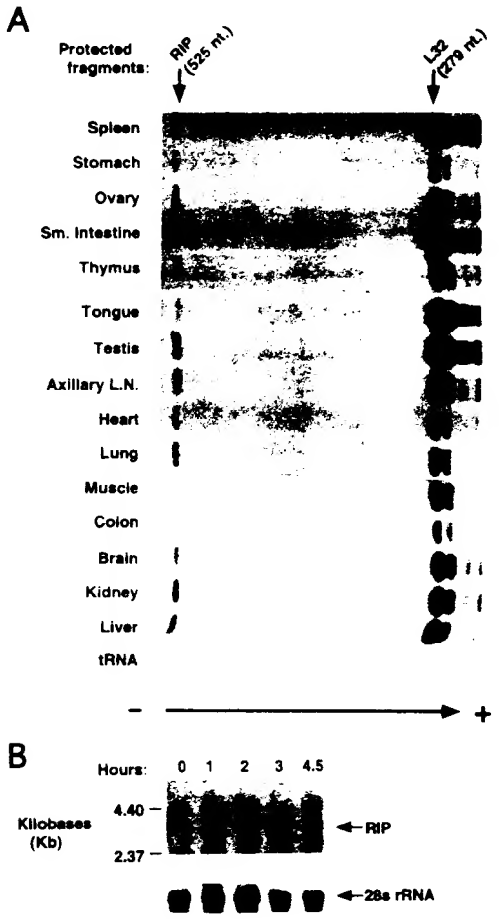

Figure 4. RIP Expression

(A) RNase protection analysis of total RNA harvested from adult tissues. Each RNA (10 μg) was incubated with labeled antisense RNAs complementary to the 3' end of the RIP cDNA sequence and to sequences within the mRNA for the ribosomal large subunit protein L32 (normalization control). Following digestion of the unhybridized RNA, the protected fragments were separated on a 6% denaturing polyacrylamide gel. tRNA (50 μg) was substituted for the sample RNA to assess the adequacy of RNase digestion.

(B) T cell activation induces RIP mRNA. An RNA blot prepared from 8 μg samples of total RNA from splenocytes treated with ConA for the indicated period of time was hybridized with labeled RIP cDNA or a 28S rDNA probe and washed as described in the Experimental Procedures.

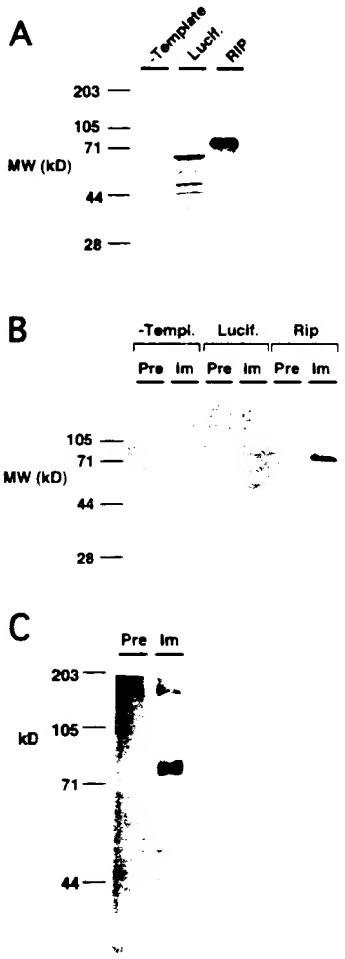

Figure 5. Immunoprecipitation of RIP

(A) An insert fragment from RIP cDNA clone III2 was transcribed and translated in vitro, and the reaction products were analyzed on a 10% SDS–polyacrylamide gel. Included as controls were samples in which either no RNA, or an RNA encoding P. pyralis luciferase, was translated

(B) Immunoprecipitation of the in vitro translation products of (A) with an antiserum raised against the C-terminus of RIP, or with preimmune serum.

(C) Immunoprecipitation of an [^{35}S]Met-labeled lysate of NIH 3T3 cells with the antiserum described in (B).

revealed the presence of a single protein species with the same molecular mass as that revealed by in vitro translation of RIP (Figure 5C)

D tection of RIP in Transiently Transfected BHK Cells

To determine whether RIP protein could have a direct effect on cell viability, BHK cells grown on coverslips were transiently transfected with an epitope-tagged version of RIP (RIP-Myc) and reacted with anti-RIP antiserum or an anti-Myc monoclonal antibody. Weak expression was detected with both antibodies. The pattern of immunoreactivity was heterogen ous, with both diffuse cytoplasmic as well as punctate perinuclear patterns observed (Figures 6A and 6B). DNA staining with Hoechst 33258 showed that many of the RIP-expressing cells had apoptotic nuclei, a feature not seen when vector or Fas control expression plasmids were used (data not shown). However, a number of RIP-expressing cells could be found that had normal-appearing nuclei; conversely, apoptotic cells having no detectable RIP staining were also seen.

The concordance of cell death and RIP expression suggested the ability to detect RIP-Myc protein might be compromised by the death of the cells in which it was being expressed. To test this and the possible role of individual RIP domains in apoptosis, two additional pitope-tagged constructs w re prepared: one lacking C-t rminal sequences, including the death domain (RIP-MycΔd ath),

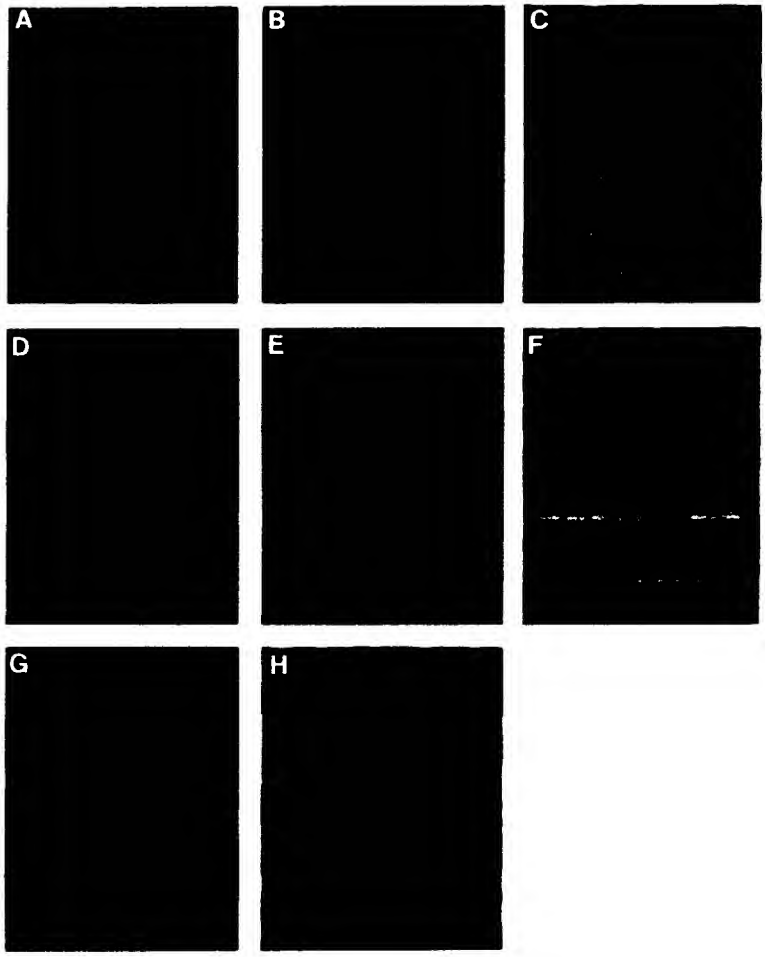

Figure 6. RIP Immunofluorescence

BHK cells were transiently transfected with plasmids expressing RIP-Myc (A–C), RIP-MycΔkinase (D–F), or RIP-MycΔdeath (G–H) and reacted with anti-Myc monoclonal antibodies (A, D, and G), anti-RIP polyclonal antibodies (B and E), or Hoechst 33258 (C, F, and H). Cells transfected with RIP-MycΔdeath plasmid did not react with anti-RIP antisera (data not shown).

and one lacking ~200 amino acids in the kinase domain (RIP-MycΔkinase). Both deletion mutants showed greater immunoreactivity with anti-Myc antibodies (Figures 6D and 6G) than the full-length construct. Only RIP-MycΔkinase was detected by the anti-RIP antiserum (Figure 6E), as expected from the deletion of its epitope from RIP-MycΔdeath.

RIP Overexpression Leads to Cell Death

To determine whether RIP was inducing cell death, we marked the transfected cells by cotransfection with β-gal. Cells were transfected with pairs of expression plasmids encoding RIP-Myc and β-gal (Price et al., 1987) at a 1:3 ratio of β-gal plasmid to RIP plasmid. After histochemical detection of β-gal activity, cells transfected with β-gal and RIP-Myc expression plasmids were found to contain a large proportion of intensely staining, shrunken blue cells that exhibited membrane blebbing and loss of adherence (Figures 7A and 7E). By contrast, transfection with β-gal, either alone (Figure 7D) or in combination with RIP-MycΔdeath (Figure 7C), had no adverse effect upon nuclear morphology and resulted in a predominantly cytoplasmic β-gal staining pattern. Cotransfection of β-gal with RIP-MycΔkinase likewise gave cytoplasmic staining of healthy-appearing cells, although a number of shrunken, blebbed cells were also seen (Figure 7B).

To quantitate these results, cells from three independent transfections were examined, and the morphologically apoptotic blue cells were enumerated as a fraction of total blue cells (Table 1). Over 57% of blue cells arising from cotransfection of RIP-Myc and the β-gal showed morphological changes consistent with apoptosis, whereas only 1%–2% of blue cells that had been transfected with β-gal plasmid or in combination with RIP-MycΔdeath exhibited such a phenotype. However, a consistent frequency of apoptotic changes was seen in cotransfections involving RIP-MycΔkinase, with almost 11% of the blue cells appearing to have undergone cell death. The fraction of cells showing morphological changes was positively correlated with the ratio of RIP-Myc or RIP-MycΔkinase plasmid to β-gal plasmid, so that increasing RIP:β-gal plasmid ratios gave higher percentages of dead blue cells (data not shown).

Additional transfection experiments were performed using cells derived from the human embryonic kidney 293 cell line (Pear et al., 1993). As with the previous assay, cell death was observed in these cells when either the RIP-Myc or RIP-MycΔkinase plasmid was used. DNA frag-

Figure 7. Apoptotic Changes in Cells Transfected with RIP and β-Gal Expression Plasmids

BHK cells were transfected on coverslips, fixed after 12 hr, and incubated in a buffer containing X-Gal to visualize β-gal activity. Transfections were carried out with plasmids encoding: (A) RIP-Myc and β-gal, (B) RIP-MycΔkinase and β-gal, (C) RIP-MycΔdeath and β-gal, or (D) β-gal alone. (E) Higher magnification of cells shown in (A).

Table 1. Quantitation of RIP-Induced Cell Death

Construct	Percent Dead ± SEM (number of cells scored)
RIP-Myc plus β-gal	57.3 ± 1.7 (618)
RIP-MycΔkinase plus β-gal	10.9 ± 1.1 (660)
RIP-MycΔdeath plus β-gal	2.0 ± 0.7 (661)
β-gal alone	1.6 ± 0.2 (639)

The cotransfections described in Figure 7 were repeated in triplicate, and the cells were fixed and analyzed for *lacZ* expression 16 hr after transfection as detailed in the Experimental Procedures. The data are expressed as the mean percentage of blue cells exhibiting signs of apoptosis as a fraction of the total number of blue cells counted (shown in parentheses).

m ntation, a hallmark of apoptotic cell death, was detected in situ in cells transfected with these plasmids using the m thod of TdT-mediated dUTP–biotin nick end labeling (TUNEL; Gavrieli et al., 1992); no significant cell death or DNA fragmentation was observed with the RIP-MycΔd ath or vector plasmids (data not shown).

Discussion

In this study, a genetic selection in yeast for proteins capable of interacting with the intracellular domain of Fas/APO-1 identified cDNAs encoding two proteins from a human T cell cDNA library: Fas itself and a novel kinase-related protein, RIP, containing a death domain. Neither protein interacts with a humanized variant of the murine *lpr*^{cg} allele, a point mutant that confers much the same phenotype on affected mice as does the original *lpr* allele (Matsuzawa et al., 1990). The finding that the intracellular domain of the *lpr*^{cg} variant is incapable of interacting with the wild-type Fas cytoplasmic domain in yeast suggests that oligomerization of Fas mediated by cytoplasmic sequences may be a prerequisite for activity. However, it has not been established whether oligomerization of Fas is necessary for interaction with RIP, or whether the same subdomain exploited for oligomerization is also required for heteromeric association with RIP. The discovery of the Fas–Fas interaction in a library screen is consistent with a r cent d monstration that TNFR1 and Fas are capable of both self- and cross-association in yeast (Boldin t al., 1995). In accord with the notion that the death domain may itself mediate such interactions, we find here that RIP can also associate with the intracellular domain of TNFR1.

Despite conservation of sequence, structure, and function between Fas and TNFR1, downstream effects of the two receptors have been dissociated in some cell types (Wong and Goeddel, 1994; Schulze-Osthoff et al., 1994), whereas responses in other cells suggest that elements of their signaling pathways may be shared (Clement and Stamenkovic, 1994; Schulze-Osthoff et al., 1994). The finding that RIP interacts with both Fas and TNFR1 in yeast is attractive for its intimation of a simple explanation for the shared manifestations of receptor ligation.

Deletion analysis of Fas has previously demonstrated that the 15 C-terminal amino acids are not required for signaling, but that larger deletions abolish the ability of Fas to induce apoptosis (Itoh and Nagata, 1993). Consistent with these findings, this study has shown that in yeast RIP binds well to a Fas molecule lacking the 16 C-terminal amino acids, but that larger deletions of the Fas cytoplasmic domain greatly reduce or abolish this interaction.

The human RIP fragment identified by yeast interaction was used to clone a murine cDNA that appears to span the entire coding region. The authentic 5′ and 3′ ends of the RIP mRNA have yet to be defined, however. Sequencing of the 3′ ends of cDNA clones III1, III2, and I1b yielded about 1.4 kb of putative untranslated sequence including an AAUAAA transcription termination signal, but no poly(A) tail (data not shown). Genomic sequence obtained from the 5′ end suggests the presence of at least one more noncoding 5′ exon (data not shown). Of the discrepancy between the 3.4 kb of identified sequence and the approximately 3.8 kb of RNA identified in blot hybridization experiments, the fraction that can be attributed to poly(A) is uncertain.

RIP mRNA is expressed at low levels in all tissues. A pattern of widespread expression has also been observed for Fas (Leithauser et al., 1993, B. Z. S. and P. L., unpublished data), although in contrast with RIP, *fas* mRNA is found at higher levels in thymus, liver, lung, and heart. Like *fas* mRNA, RIP mRNA is induced in splenocytes after activation with ConA. Although a r quirement for RIP in Fas-mediated killing has not be n established, coordinate induction of Fas and RIP may contribute to increased sus-

ceptibility of T cells to Fas-mediated cell death following activation (Owen-Schaub et al., 1992).

The N-terminus of RIP has strong homology to kinases of both Ser/Thr and Tyr kinase subfamilies. In the two interacting loops that appear to control hydroxyamino acid recognition (Hubbard et al., 1994; Taylor and Radzio-Andzelm, 1994), RIP closely resembles a Ser/Thr kinase. In particular, it lacks the Ala–X–Arg or Arg–X–Ala motif in subdomain VI and the Pro–X–X–Trp motif in subdomain VIII that closely correlate with Tyr substrate specificity (Hanks et al., 1988). However, among sequences available at the time of submission, RIP shows the greatest global similarity to murine *lck*, a Tyr kinase of the Src family. The relatedness to Tyr kinases is especially apparent among the framework residues outside the putative active site. For example, RIP has a tryptophan at position 269 that is present in all Tyr kinases analyzed by Hanks and Quinn (1991), but absent from all Ser/Thr kinases examined except Mos. The presence of structural motifs from both Tyr and Ser/Thr kinases has also been noted for the soybean kinase GmPK6 (Feng et al., 1993), with which RIP shares high global similarity. RIP contains a small number of amino acids that differ from the conserved residues of either Ser/Thr or Tyr kinases, for example a Gly at position 24 that is present in the majority of kinases from both classes. However, at residues that are conserved in all protein kinases, RIP follows the consensus.

We have found that overexpression of RIP results in the induction of a cell death program morphologically similar to apoptosis. Deletion of the C-terminal region of RIP spanning the segment of death domain homology eliminated the apoptotic response, but deletion of the kinase domain did not entirely quench activity. It may be that the overexpression of the death domain itself is lethal to transfected cells, consistent with the observation by Boldin et al. (1995) that expression of the free intracellular domain of TNFR1 has a cytocidal effect on HeLa cells. The induction of apoptosis by transient transfection suggests that the level of expression or ability of death domain–bearing proteins to self-associate may be subject to some form of regulation to prevent the spontaneous initiation of apoptosis in the absence of ligand or other eliciting stimulus.

The notion that a stoichiometrically limiting intracellular effector is involved in the pathway of Fas-mediated apoptosis is supported by the recent demonstration that maximal signaling by Fas and the TNF receptors requires an intermediate level of receptor cell surface expression (Clement and Stamenkovic, 1994). This result is consistent with a model in which the initiation of signal transduction is critically dependent on the aggregation-mediated focal accumulation of a limiting cytoplasmic ligand, which can be diluted to the point of inefficacy by overexpression of the c ll surface receptor. The data presented here that RIP is not encoded by a highly expressed RNA, but that the transcript accumulates rapidly in response to T cell activation, in turn admits the possibility that RIP may be limiting for receptor-mediated apoptosis. However, other factors may also interact with the Fas intrac llular domain, as sugg sted by the findings that Fas can transmit activation as w ll as death signals (Alderson et al., 1993).

Still unclear is the mechanism by which formation of a multimeric Fas complex would result in signal transduction. Although enzymatic activity has not been demonstrated for RIP, the possibility that it encodes a protein kinase is attractive given the proposed participation of at least one Tyr kinase in Fas signaling (Eischen et al., 1994). However, results with the mutant plasmid RIP-MycΔkinase suggest that any kinase potential RIP has may not be required for its induction of cell death.

In preliminary experiments, a monoclonal antibody recognizing murine Fas has been found to specifically coimmunoprecipitate a polypeptide of about 59 kDa that reacts with anti-RIP antiserum (data not shown). This 59 kDa protein may represent a proteolytic fragment of RIP or a related protein that is recognized by anti-RIP antibodies. Although the evidence that RIP binds to Fas in yeast favors a model in which RIP acts directly downstream of Fas in a death pathway, it is also possible that RIP has other actions, or that the normal physiological role of the molecule lies in a pathway initiated by other cues. The creation of mice or cell lines homozygously deficient in RIP should help to address this point. In addition, the elucidation of any target(s) of action of RIP may also facilitate our understanding of the role(s) RIP plays in the cell death response.

Experimental Procedures

Plasmid Construction

The yeast interaction system was modified from that described by Gyuris et al. (1993) by engineering the LexA expression plasmid to remove an internal MluI site and to insert MluI and NotI sites downstream from the DNA portion encoding the C-terminus of the gene. The resulting distal polylinker has the site sequence MluI–PmeI–NotI–EcoRI in the frame in which the MluI site encodes Thr and Arg (frame 1). Complete sequences are available from the authors (B. S.) upon request.

Receptor cytoplasmic tails were amplified by PCR from cDNA libraries and cloned as MluI–NotI or BssHII–NotI fragments using the following oligos: Fas, 5′-CGC GGG ACG CGT AAG GAA GTA CAG AAA ACA TGC-3′ and 5′-CGC GGG GCG GCC GCT CTA GAC CAA GCT TTG GAT TTC-3′; TNFR1, 5′ CGC GGG GCG CGC TAC CAA CGG TGG AAG TCC AAG-3′ and 5′ CGC GGG GCG GCC GCT GCC CGC AGG GGC GCA GCC TCA-3′; TNFR2, 5′-CGC GGG ACG CGT AAG AAG CCC TTG TGC CTG CAG-3′ and 5′-CGC GGG GCG GCC GCT TTA ACT GGG CTT CAT CCC AGC-3′. The Fas cytoplasmic domain used in the library screen diverges at the Glu 5 residues prior to the C-terminus and continues an additional 25 residues through vector sequences to the C-terminus. In all subsequent analysis, these residues were found not to contribute detectably to either Fas–Fas or Fas–RIP interaction. A mutant Fas bait protein analogous to the *lpr*^{cg} point mutation was made by mutating the Val at position 254 of human Fas to Asn using the following oligos in a recombinant PCR reaction: 5′-CGA AAG AAT GGT AAC AAT GAA GCC-3′ and 5′-GGC TTC ATT GTT ACC ATT CTT TCG-3′. In the resulting construct, residues 330 and 331 were also converted from Glu and Ile to Gly and Asn, respectively. Fas deletion mutants were made by PCR with the Fas 5′ oligo and 3′ oligos substituting a stop codon for the amino acid at the indicated position. products were subcloned into the LexA expression plasmid as described above. Oligos used were the following: (S320*) 5′-CGC GGG GCG GCC GCT TTA AGT AAT GTC CTT GAG GAT GAT-3′; (K299*) 5′-CGC GGG GCG GCC GCT TTA GAG ATC TTT AAT CAA TGT GTC-3′; (H285*) 5′-CGC GGG GCG GCC GCT TTA AAG TTG ATG CCA ATT ACG AAG-3′; (K263*) 5′-CGC GGG GCG GCC GCT TTA GAT CTC ATC TAT TTT GGC TTC-3′.

A Myc-tagged version of RIP (RIP-Myc) was made by digesting RIP clone III2 with TfiI and ligating a HindIII–TfiI adaptor to the 5′ end and a TfiI–NotI adaptor containing the Myc epitope and a stop codon to the 3′ end. The sense (145) and antisense (146) oligonucleotides com-

prising the 3′ adaptor were as follows: 145, 5′-ATT CGT GCC AGC CAG AGC GGC ATG GAG CAG AAG CTC ATC TCA GAA GAA GAC CTC GCG TAA GC-3′ and 146, 5′-GGC CGC TTA CGC GAG GTC TTC TTC TGA GAT GAG CTT CTG CTC CAT GCC GCT CTG GCT GGC ACG-3′. The resulting insert was cloned into the HindIII and NotI sites of pcDNA I (Invitrogen). To make RIP-MycΔkinase, a PCR reaction was performed using a 5′ RIP primer (160, 5′-CCC AAG CTT GTT GGA GAT TCT GAG CAA TC-3′) and an internal kinase domain primer (161, 5′-CCC GAT CTG CAG GTC ATG TAA GTA GCA CAT GCC-3′). The resulting product was cloned into the HindIII and PstI sites of RIP-Myc resulting in the deletion of RIP residues 132–323. RIP-MycΔdeath was made by PCR using a T7 primer and a Myc tag–containing primer (151, 5′-CCC CTC GAG TTA GAG GTC TTC TTC TGA GAT GAG CTT TTG CTC TTT CTT TAA ACT TGC CAC-3′). The amplified RIP-MycΔdeath sequence, lacking amino acids 309–656, was subcloned as a HindIII–XhoI fragment into pcDNA I. Myc tags were located at the C-terminus of all proteins, and thus, detection of the Myc epitope requires translation of the entire cloned sequence. The BAG retrovirus vector encoding β-gal has been described (Price et al., 1987).

Yeast Strains and Library Screen

Yeast transformation with library DNA was performed by the method of Schiestl and Gietz (1989) as follows. Recipient cells, EGY48/pRB1840 (Gyuris et al., 1993) bearing LexA–fusion protein plasmids, were grown overnight in YPAD medium to a density of approximately 10^7 cells/ml, then diluted in 100 ml of warmed YPAD to a density of 2×10^6 cells/ml and regrown to 10^7 cells/ml. The cells were harvested and washed in water, resuspended in 1 ml of water, transferred to a sterile microcentrifuge tube, and pelleted. The pellet was resuspended in 0.5 ml of 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 M Li acetate (pH adjusted to 7.5 with acetic acid and passed through 0.2 μ filter). 50 μl of the resulting suspension was mixed with 1 μg of transforming DNA and 50 μg of single-stranded salmon sperm DNA, after which 0.3 ml of a solution of 40% polyethylene glycol-4000 in Tris, EDTA, LiOAc was added and mixed thoroughly, followed by incubation at 30°C with agitation for 30 min. After a heat pulse at 42°C for 15 min, the cells were pelleted in a microcentrifuge, and the pellets were resuspended in 1 ml of Tris, EDTA, diluted, and plated on selective medium. Library screening and recovery of plasmids were performed as described by Gyuris et al. (1993).

For assessing the interaction of RIP and Fas with other bait proteins, cells of the yeast strain EGY48/pSH18-34 were transformed with the indicated bait construct and selected on Ura$^-$ His$^-$ glucose plates. These bait strains were subsequently used for transformation of the RIP or Fas library plasmids and plated on Ura$^-$ His$^-$ Trp$^-$ glucose plates. Several colonies from each bait/interactor combination were picked and plated in duplicate on Ura$^-$ His$^-$ Trp$^-$ X-Gal plates containing either 2% glucose or 2% galactose, 1% raffinose. The LexA–TNFR2 bait strain gave a weak blue color reaction when grown on galactose, indicating spontaneous transcriptional activation by the fusion protein.

β-Gal Assays

Assays of crude extracts were carried out as described (Rose et al., 1990). Cells bearing the appropriate bait and interaction plasmid were grown to saturation overnight at 30°C in minimal Ura$^-$ His$^-$ Trp$^-$ medium with 2% glucose. The next day, cells were diluted 1:50 into medium containing 2% galactose and 2% raffinose and allowed to grow overnight. Cells were spun and resuspended in breaking buffer (100 mM Tris [pH 8], 20% glycerol [v/v], 1 mM DTT). Cells were lysed in the presence of 10 mM PMSF by vortexing with acid-washed beads. Lysates were cleared by centrifugation, and an aliquot was incubated in Z buffer (Miller, 1972) with 0.67 mg/ml o-nitrophenyl-β-D-galactoside substrate. Reactions were stopped with Na_2CO_3 when an appropriate level of color had developed. Protein concentrations were determined by Bradford assay. β-Gal units were calculated by the equation: specific activity (nmoles/min/mg) = $(OD_{420} - 378)$/(time [min] × vol [ml] × concentration [mg/ml]).

cDNA Cloning

Additional clones overlapping the primary RIP isolate were sought in two libraries: an expression library prepared in the CDM8 plasmid vector using mRNA isolated from the human cytolytic T cell line WH3 and a commercially available human leukocyte library in λ phage purchased from Clontech (HL1169a). Both libraries were screened by filter replica hybridization, using radiolabeled probes derived from the insert isolated by interaction screening, as well as from subsequent inserts identified by hybridization.

To isolate murine RIP cDNA clones, 1.2 kb of human RIP sequence was subcloned into two halves and radiolabeled using the random hexamer method (Feinberg and Vogelstein, 1983). An oligo(dT)-primed mouse thymus cDNA library from a (C57Bl/6 × CBA)F1 mouse (Stratagene) was plated out and 10^6 plaques were screened using each of the human fragments on duplicate GeneScreen filters (DuPont). Hybridization conditions were 5 × SSPE, 10 × Denhardts, 2% SDS, 0.1 mg/ml herring sperm DNA at 55°C overnight. Filters were washed in 2 × SSC, 0.1% SDS at 55°C with several changes over 1 hr. Plaque purified phage were isolated with three rounds of screening (Sambrook et al., 1989), and in vivo excision was carried out using Exassist phage and SOLR recipient cells (Stratagene). Seven independent clones were isolated that fell into the four classes in Figure 2A. The coding sequence of RIP was obtained as a composite from the cDNA clones sequenced on both strands using Sequenase T7 polymerase (United States Biochemical). Several nucleotide polymorphisms were detected between the multiple clones, only one of which resulted in an amino acid difference: a Thr to Ile at position 473. Sequence comparisons were done with Genetics Computer Group, Inc. or MegAlign (DNAStar, Inc.) software using default parameters.

RNase Protections and RNA Blot Hybridization

Tissue RNA samples were prepared from wild-type FVB mice (Taconic) by guanidinium thiocyanate lysis and centrifugation through a CsCl cushion (Chirgwin et al., 1979). An antisense probe for RIP made from cDNA clone III1 linearized with SpeI was synthesized using T7 polymerase with an in vitro transcription kit (Stratagene), with the addition of 20 μM cold rUTP and 100 μCi [^{32}P]UTP (New England Nuclear). The ribosomal L32 probe was synthesized from an XbaI-linearized template at 10% the specific activity of the RIP probe. The use of L32 as an internal control for RNA loading has been described elsewhere (Shen and Leder, 1992). The in vitro transcription products were slightly larger than the protected fragments of 525 nt and 279 nt, respectively, as expected. RNase protection was carried out as described by Melton et al. (1984).

For measuring RIP in activated T cells, spleens were removed from adult animals and dissociated using a stainless steel mesh. Cells ($\sim 10^7$ per timepoint) were exposed to ConA (10 μg/ml) for 0–4.5 hr, and RNA was prepared as described above. Approximately 8 μg of total RNA from each sample was loaded on a 0.9% agarose, 4% formaldehyde gel, electrophoresed, and transferred to nylon filters (GeneScreen, DuPont) by capillary transfer (Sambrook et al., 1989). The blot was hybridized with either an RIP probe corresponding to the C-terminal half of the protein (cDNA III1) or with a probe detecting ribosomal 28S RNA and incubated overnight in 40% formamide, 4 × SSC, 10% dextran sulfate, 7 mM Tris (pH 7.6), and 20 μg/ml salmon sperm DNA at 42°C. Blots were washed at 50°C in 1 × SSC, 0.1% SDS (RIP probe) or 60°C in 0.1 × SSC, 0.1% SDS (28S probe).

RIP Antisera and Immunoprecipitations

Rabbit polyclonal antisera recognizing RIP were prepared by Pocono Rabbit Farm and Laboratory, Inc. using a fusion protein containing the C-terminal 250 amino acids of mouse RIP fused to maltose-binding protein (MBP, New England Biolabs). MBP–RIP was purified by amylose chromatography and acrylamide gel elution. For immunoprecipitations, RIP was first transcribed in vitro using 15 μg of full-length RIP template in a reaction containing 3.3 mM each ATP, GTP, CTP, and UTP, 280 U of RNase inhibitor, 400 U of Sp6 RNA polymerase, and 1 × Sp6 buffer (Boehringer Mannheim Biochemicals). Translations were carried out using a reticulocyte lysate kit containing a luciferase positive control (Promega) using approximately 4 μCi [^{35}S]Met per reaction. Reaction products were diluted to 1 ml in a modified RIPA buffer (0.5% NP-40, 0.5% sodium deoxycholate, 0.025% SDS, 50 mM Tris, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate [pH 7.6]) containing protease inhibitors and precleared with normal rabbit serum and protein A–Sepharose. Reactions were split in half, 5 μl of preimmune or immune serum was added, and samples

were allowed to rotate for 1 hr at 4°C. Complexes were precipitated with 40 μl of protein A–Sepharose (1:1), washed with modified RIPA buffer, and resolved by SDS–PAGE.

For immunoprecipitation from metabolically labeled cells, a 150 mm plate of subconfluent NIH 3T3 cells was incubated overnight in Met-deficient DMEM (GIBCO/BRL) supplemented with [^{35}S]Met (~100 μCi/ml), 5% dialyzed fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were lysed for 15 min on ice in lysis buffer (250 mM NaCl, 50 mM HEPES–KOH, [pH 7.5], 5 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF, 0.1% Triton-X, 100 μg/ml PMSF, 2 μg/ml pepstatin, and 10 μg/ml each aprotonin and leupeptin), scraped, and spun at 15,000 × g for 10 min. Cleared lysate was precleared as above, followed by addition of antibodies (3 μl of preimmune or immune rabbit serum). Immune complexes were harvested with protein A–Sepharose, and beads were washed with lysis buffer before resuspension in denaturing sample buffer. Following SDS–PAGE, the gel was enhanced with enlightening solution (DuPont) and dried for autoradiography.

Transfections and Immunofluorescence

BHK cells were plated the night before transfection at a density of 10^4 cells per 18 mm round coverslip (VWR Scientific). $CaPO_4$ precipitates were made, and cells were reacted with antibodies for immunofluorescence as described by Heald et al. (1993). In most cases, cells were analyzed 18–20 hr after transfection. The 9E10 anti-Myc antibody (Evan et al., 1985) was obtained as an ascites from the Harvard Cell Culture facility and used at 20 μg/ml. RIP antiserum was used at a dilution of 1:200. FITC anti-mouse and Texas red anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch. Cells were examined with a Zeiss Axiophot fluorescent microscope.

β-Gal activity in cells was visualized by fixing cells with 0.5% glutaraldehyde for 15 min followed by extensive washing in PBS with 5 mM $MgCl_2$. Cells were stained in PBS containing 20 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) until a suitable color developed, usually for 2–3 hr. To enumerate the fraction of blue cells that had undergone apoptotic changes, cells were transfected with one of the four plasmid combinations described and fixed 16 hr after transfection. Blue cells were included for analysis only if their morphological status could be scored unambiguously.

Acknowledgments

We are grateful to Roger Brent and Jeno Gyuris for their generous contributions of reagents and support throughout this work and Waldemar Kolanus for his assistance in setting up and carrying out the yeast screen. We thank Jon Madison for help with the yeast β-gal assay and Connie Cepko for use of the BAG vector. We appreciate the generous assistance of Frank McKeon and his laboratory in performing transfections. In addition, we thank Yasumasa Ishida, David Chan, Tim Lane, Michelle Kelliher, and other members of the Leder lab for discussions and advice, as well as Nancy Chiu and Felipe Pimentel-Muinos in the Seed lab for experimental assistance. This work was supported in part by National Institutes of Health grant DK43031. B. Z. S. is a predoctoral fellow of the Howard Hughes Medical Institute.

Received February 28, 1995; revised April 18, 1995.

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GenBank Accession Number

The accession numbers for the human and mouse RIP sequences reported in this paper are U25994 and U25995, respectively.

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AU   Wong G H; Tartaglia L A; Lee M S; Goeddel D V
TI   Antiviral activity of   ***tumor***     ***necrosis***
       ***factor***   is signaled through the 55-kDa type I   ***TNF***
     receptor [corrected] [published erratum appears in J Immunol 1993
     Jan 15;150(2):705].
SO   JOURNAL OF IMMUNOLOGY, (1992 Nov 15) 149 (10) 3350-3.
     Journal code: IFB. ISSN: 0022-1767.
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0022-1767/92/1491-3350$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 149, 3350-3353, No. 10, November 15, 1992
Printed in U.S.A.

ANTIVIRAL ACTIVITY OF TUMOR NECROSIS FACTOR (TNF) IS SIGNALED THROUGH THE 55-kDa RECEPTOR, TYPE I TNF

GRACE H. W. WONG,[1]* LOUIS A. TARTAGLIA,† MICHAEL S. LEE,* AND DAVID V. GOEDDEL†

*From the Departments of *Immunology and †Molecular Biology, Genentech, Inc., South San Francisco, CA 94080*

Agonist antibodies (Ab) to the two TNF receptors, TNF-R1 (55 kDa) and TNF-R2 (75 kDa), have been shown to signal many of the distinct functions induced by TNF-α. We have found that anti-TNF-R1, but not anti-TNF-R2, Ab trigger antiviral activity in human hepatoma Hep-G2 cells and enhance the antiviral activity of IFN-γ in human lung fibroblast A549 cells. Likewise, anti-human-TNF-R1 Ab had antiviral enhancing activity on murine L929 cells engineered to express human TNF-R1. However, L929 cells that express human TNF-R1 lacking most of the intracellular domain fail to respond to anti-human-TNF-R1 Ab. This demonstrates that the intracellular domain of TNF-R1 is necessary to generate antiviral activity. TNF-R1 but not TNF-R2 also signals killing of virus-infected cells by TNF-α. Thus, all the known antiviral activities of TNF-α are mediated through TNF-R1.

TNF-α has many physiologic effects including antiviral actions (1–5). It can act alone and in a synergistic manner with IFN-γ to protect cells from viral infection (5–7). TNF-α also selectively kills virus-infected but not uninfected cells (5, 8–10). Moreover, TNF-α sensitizes virus-infected cells to stresses such as heat and radiation, while enhancing the resistance of uninfected cells (11).

TNF-α binds two types of cell surface receptor molecules, referred to as TNF-R1 (55 kDa) and TNF-R2 (75 kDa) (12–16). Some cells seem to express only one receptor type, whereas most express both (12–16). The two receptors are about 30% identical in their extracellular ligand binding domains, but are dissimilar in their cytoplasmic portion (12–16), suggesting that they mediate different intracellular signals. Indeed, different activities of TNF-α are differentially elicited by activating either TNF-R1 or TNF-R2 (17–20). Recent reports have shown that agonistic Ab[2] to TNF-R1 can trigger many responses normally induced by TNF-α including cytotoxicity, cell proliferation, and induction of cellular genes (17–20). Similar Ab to TNF-R2 have been shown to trigger proliferation in thymocytes and a cultured T cell line (20). Anti-TNF receptor Ab are thought to mimic TNF-α effects by cross-linking the receptors (17). It is not clear which TNF receptor is responsible for the antiviral activities of TNF-α or if those effects can be induced by anti-receptor Ab. To address these questions, we have examined the antiviral activities of known agonist Ab to TNF-R1 and TNF-R2.

MATERIALS AND METHODS

Cell lines. These were originally obtained from the American Type Culture Collection and were free of *Mycoplasma*. The Hep-G2, A549, and L929 cell lines were grown in MEM, F12, and DMEM media, respectively. All media were supplemented with 5% FCS, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were incubated at 37°C in a 5% CO_2 atmosphere. The characterization of L929.hR1.17 (L929 cells overexpressing intact human TNF-R1) and L929.hR1Δ.4 (L929 cells overexpressing a mutant human TNF-R1 lacking 182 amino acids of its intracellular domain) has been described previously (21).

Reagents. Recombinant human TNF-α (4×10^7 U/mg), murine TNF-α (5×10^7 U/mg), and recombinant human IFN-γ (5×10^7 U/mg) were supplied by the Genentech manufacturing group. The rabbit antisera against murine TNF-R1, murine TNF-R2, and nerve growth factor were the same as those described previously (20) and were a gift from G. Bennett (Genentech). Polyclonal Ab and mAb specifically directed against the human TNF receptors have also been described (21, 22). Twelve-affinity purified mAb against either human TNF-R1 (981, 982, 983, 984, 985, 986, 993, 994, 1002, 1011, 1015, and 1021) or human TNF-R2 (1035, 1036, 1037, 1038, 1039, 1040, 1043, 1044, 1045, 1046, 1047, and 1048) were obtained from B.M. Fendly (Genentech).

Antiviral assay. A suspension of cells (100 μl) at 2×10^5/ml in MEM supplemented with 5% FCS was added to each well of a 96-well plate for 24 h before the assay. The test samples (50 μl) were added to the attached cells in the first column and the samples were serially twofold diluted. After 24 h, cells were challenged with virus (either VSV or EMCV) diluted in MEM with 2% FCS at multiplicities of infection of 0.1 to 2.0 and were further incubated at 37°C. After 24 h, virus control wells were checked by microscopic examination to confirm 90 to 100% lysis. The fluid from all wells was poured off and the attached viable cells were stained with 0.5% crystal violet in 20% methanol for 10 to 15 min at ambient temperature. Cell viability was determined by eluting the dye from the stained cells with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol and measuring A_{540} (21).

Killing of virus-infected cells. Cells (4×10^4/well) were infected with VSV at a multiplicity of infection of 0.1 and virus was allowed to adsorb for 1 h. The cells were then washed three times before adding the serially diluted samples of either TNF-α or antisera in F12 medium without FCS. After 12 h, the cell viability was determined as above.

RESULTS AND DISCUSSION

Antibodies to TNF-R1 have antiviral activity. To test whether the antiviral properties of TNF-α are mediated through TNF-R1 and/or TNF-R2, we assayed a series of agonist Ab for antiviral activity. These Ab have been shown to recognize either human TNF-R1 or human TNF-R2 specifically (21, 22). Pretreatment of human hepatoma Hep-G2 cells with either TNF-α or anti-TNF-R1 Ab, protected against the CPE of VSV (Fig. 1, *A* and *B*). This protective effect increased with increasing doses of either

Received for publication May 28, 1992.
Accepted for publication September 1, 1992.
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[1] Address correspondence and reprint requests to Grace H.W. Wong, Genentech, Inc., 460 Pt. San Bruno Boulevard, South San Francisco, CA 94080.

[2] Abbreviations used in this paper: Ab, antibody; VSV, vesicular stomatitis virus; EMCV, encephalomyocarditis virus; CPE, cytopathic effect.

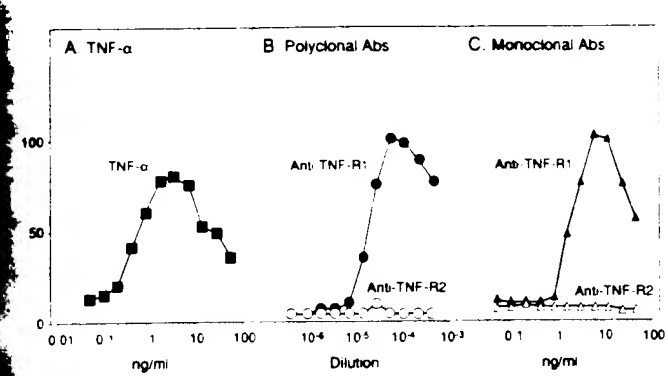


Figure 1. Anti-TNF-R1 Ab protect Hep-G-2 cells from VSV-mediated CPE. Hep-G2 cells were pretreated with TNF-α (*A*), rabbit anti-human TNF-R1 or TNF-R2 Ab (*B*), and mouse mAb against either human TNF-R1 (mixture of 12 different types, initial concentration = 0.1 μg/ml of each) or human TNF-R2 (mixture of 12 different types, initial concentration = 0.1 μg/ml of each) (*C*), and the reagents were serially twofold diluted. After 24 h, cells were challenged with VSV for 24 h, and the viable cells were stained with crystal violet as described in *Materials and Methods*.

TNF-α or Ab to a point of near complete protection. Higher concentrations diminished the protective effect of TNF-α or anti-TNF-R1 Ab. Because high doses of TNF-α and anti-TNF-R1 are not toxic to these cells, these data suggest that the high concentrations may inhibit receptor activation, perhaps by blocking multimerization of TNF-R1.

In contrast to polyclonal Ab against TNF-R1, polyclonal Ab against human TNF-R2 did not protect Hep-G2 cells from virus-mediated CPE. These anti-TNF-R2 polyclonal Ab have been shown to exert potent agonist activity in human thymocyte proliferation assays (L. A. Tartaglia, M. A. Palladino, D. V. Goeddel, unpublished observations). In addition, polyclonal Ab to mouse TNF-R1, which mimic TNF-α activity in mouse cells (20), had no protective effect in human Hep G-2 cells. This demonstrates that although mouse and human TNF-R1 extracellular domains share about 70% sequence identity, polyclonal Ab against the extracellular domain of murine TNF-R1 do not activate human TNF-R1. Control experiments demonstrate that the protective effects of the anti-human TNF-R1 antisera are due to specific Ab and not to contaminants in the rabbit immune sera. The anti-human TNF-R1 had no effect on murine cells, and its antiviral activity on human cells could be neutralized by preincubation with goat anti-rabbit Ig (data not shown).

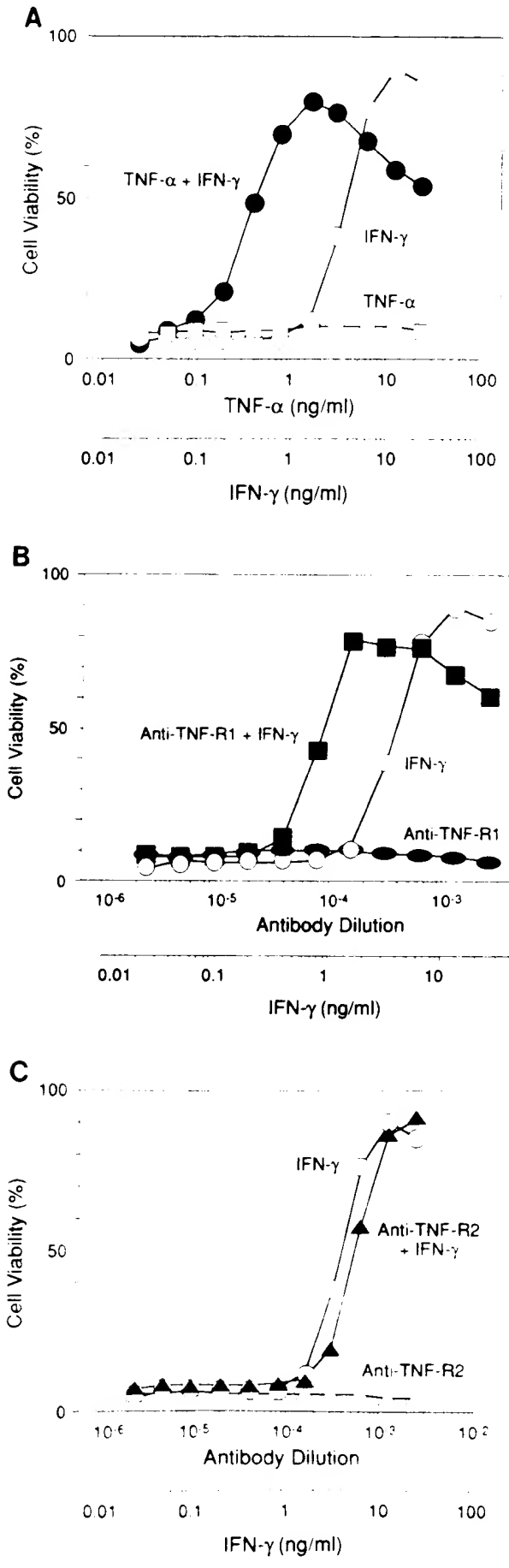
Twelve mAb to human TNF-R1 and 12 mAb to human TNF-R2 were also examined for their ability to protect Hep-G2 cells against VSV-mediated CPE. When tested individually, none had antiviral activity. However, the mixture of 12 mAb against TNF-R1 elicited antiviral activity in these cells (Fig. 1*C*). Thus, it appears that TNF-R1 may require proper orientation for receptor clustering and/or activation that are not easily fulfilled by a single mAb. Alternatively, the signal for antiviral activity may require the aggregation of more than two receptor molecules per complex. Similar results have been observed for other TNF-R1-mediated activities of TNF-α (17). No effects were seen with a mixture of 12 mAb to TNF-R2 (Fig. 1*C*), despite their ability to act as agonists in a thymocyte proliferation assay (L. A. Tartaglia, M. A. Palladino, D. V. Goeddel, unpublished observations). Hep-G2 cells express higher levels of TNF-R2 than TNF-R1 as determined by flow cytometry using polyclonal Ab (data not shown). Thus, the lack of antiviral activity of the various anti-TNF-R2 Ab is clearly not due to a lack of corresponding receptors.

TNF-R1 mediates the synergistic enhancement of IFN-γ action by TNF-α. TNF-α alone cannot protect against EMCV-mediated CPE in the human A549 cell line. However, it can greatly enhance the antiviral activity of IFN-γ in this cell line (Fig. 2*A*). Similarly, anti-TNF-R1 Ab alone do not protect A549 cells from EMCV-mediated CPE. However, in the presence of IFN-γ, polyclonal anti-TNF-R1 Ab increase the antiviral response of this cell line (Fig. 2*B*). This synergistic interaction was not observed using agonist anti-TNF-R2 Ab (Fig. 2*C*). The combination of 12 purified mAb against various epitopes of human TNF-R1 in combination with IFN-γ yielded results similar to those obtained with the polyclonal anti-TNF-R1 Ab. However, when administered one at a time, the monoclonals elicited no antiviral enhancing effects (data not shown).

Cytoplasmic domain of TNF-R1 is required for TNF's antiviral action. In murine L929 cells, neither TNF-α nor anti-murine TNF-R1 has antiviral activity alone, but both greatly enhance the antiviral activity of murine IFN-γ. Anti-human TNF-R1 Ab have no enhancing activity on this parental murine cell line (data not shown), consistent with the known species specificity of these antibodies (21). However, L929 cells engineered to express human TNF-R1 (L929.hR1.17) (21) responded to not only anti-murine TNF-R1 but also anti-human-TNF-R1 polyclonal Ab by increased resistance to VSV-mediated CPE in the presence of IFN-γ (Fig. 3*A*). These results demonstrate that the signaling machinery responsible for mediating antiviral activity in the mouse can interact with human TNF-R1. In contrast, L929 cells that express human IFN-γ receptors do not respond to human IFN-γ (23–25), demonstrating that the human IFN-γ receptor cannot activate the mouse signaling apparatus; other human components are presumably required.

An L929 cell line that stably expresses a truncated human TNF-R1 lacking the majority of the intracellular domain (L929.hR1Δ.4) (21) was also tested for responsiveness to the agonist TNF receptor Ab. Unlike the L929.hR1.17 cell line, no antiviral enhancing activity was detected with the anti-human TNF-R1 Ab. However, antiviral enhancing activity was still observed with the anti-murine TNF-R1 but not the anti-murine TNF-R2 Ab (Fig. 3*B*). The lack of activity seen with the anti-human TNF-R1 Ab was not due to inadequate receptor number since the L929.hR1Δ.4 cell line expresses higher levels of human TNF-R1 than L929.hR1.17 cells (21). The same results were obtained with other L929 clones expressing the full length and truncated human TNF-R1 (data not shown). Thus, the signaling of antiviral activity by TNF-R1 requires the TNF-R1 intracellular domain. This result is consistent with the requirement of this domain for cytotoxicity (21).

Killing of virus-infected cells by TNF-α is mediated by TNF-R1. In addition to protecting uninfected cells from virus-mediated CPE, TNF-α has also been shown to accelerate the lysis of virus-infected cells (5, 10). When assayed 12 h after VSV infection, about 75% of A549 cells are viable. However, when the VSV-infected A549 cells were treated with either TNF-α or TNF-R1 Ab, their viability decreased significantly (Fig. 4). No such decrease



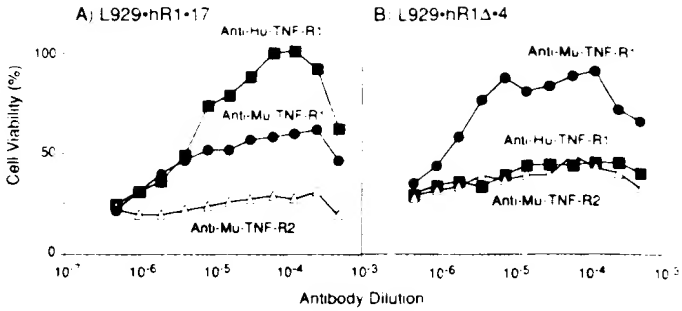


Figure 3. Anti-human TNF-R1 Ab enhance the antiviral effect of murine IFN-γ in mouse cells engineered to express human TNF-R1. L929.hR1.17 cells that overexpress human TNF-R1 (*A*) or L929.hR1Δ4 cells that overexpress truncated human TNF-R1 (*B*) were pretreated with rabbit polyclonal Ab against human TNF-R1, murine TNF-R1, or murine TNF-R2 in the presence of murine IFN-γ (0.1 ng/ml) for 7 h before VSV infection. After 24 h, percent cell viability was determined as described in *Materials and Methods*.

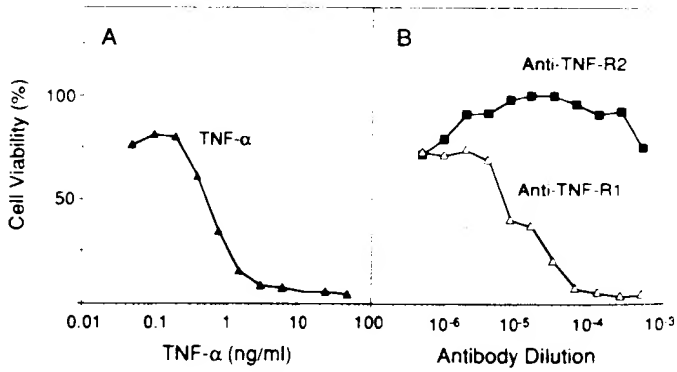


Figure 4. Polyclonal Ab against TNF-R1 kill VSV-infected A549 cells. VSV-infected cells were challenged with TNF-α (*A*) or polyclonal agonist Ab against human TNF-R1 or TNF-R2 (*B*), and cell viability was determined as described in *Materials and Methods*.

in viability was observed in response to anti-TNF-R2 Ab (Fig. 4). Other control Ab (anti-nerve growth factor, anti-murine TNF-R1, and anti-murine-TNF-R2) also had no effect on the viability of VSV-infected A549 cells (data not shown).

TNF-α combats viral infection in at least two ways: by inducing resistance to viral infection in uninfected cells (4, 5, 10) and by selectively killing virus-infected cells (5, 8–10). Unfortunately, high levels of TNF-α are toxic in vivo (1, 2), and therefore it has been difficult to use TNF-α as a specific therapeutic agent. Our results show that TNF-α's antiviral activity may be mediated entirely by TNF-R1. It is possible that part of TNF-α's toxicity in vivo may be mediated by TNF-R2. This possibility is strengthened by the finding that human TNF-α (which binds to only mouse TNF-R1) is less toxic to mice than murine TNF-α (which binds both TNF-R1 and TNF-R2) (26, 27). Therefore, it is possible that specific agonists of TNF-R1 may retain full antiviral activity while causing less toxicity (since the spectrum of activities signaled by TNF-R2 will not be induced). Additional experiments will reveal which of TNF's other effects are transduced by TNF-R1 or TNF-R2. This may facilitate development of TNF ther-

Figure 2. Anti-TNF-R1 but not anti-TNF-R2 Ab enhance antiviral activity of IFN-γ in A549 cells. Cells were pretreated with TNF-α (*A*), polyclonal Ab against human TNF-R1 (*B*), or against human TNF-R2 (*C*) in the presence or absence of human IFN-γ for 24 h before adding EMCV. Protection against virus-mediated cell killing was assessed as in *Materials and Methods*.

ples that separate undesirable toxic effects from beneficial activities.

Acknowledgments. We thank the manufacturing roup at Genentech for providing pure recombinant human cytokines; Greg Bennett and Brian Fendly for polyonal Ab and mAb, respectively; Louis Tamayo for art ork; and Sunita Sohrabji for help in preparing the manscript. Grace H.W. Wong thanks Dr. Sasha Kamb (Gau ai) for support, advice and comments on the manuscript.

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published erratum that appears in **J. Immunol. 1993 Jan 15;150(2):705**

0022-1767/93/1502-705$0
The Journal of Immunology

Vol. 150, 705, No. 2, January 15, 1993
Printed in U.S.A.

ERRATUM

In the November 15, 1992 issue of the *Journal*, Volume 149, Number 10, page 3350, an error was made in the title of the article by Grace H. W. Wong, Louis A. Tartaglia, Michael S. Lee, and David V. Goeddel. The correct title should read: "Antiviral Activity of Tumor Necrosis Factor Is Signaled through the 55-kDa Type I TNF Receptor."

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From: Romeo, David
Sent: Tuesday, November 10, 1998 4:15 PM
To: STIC-ILL
Subject: 08/981,559 Boldin M P; Mett I L; Varfolomeev E E; Chumakov I; Shemer-Avni Y;

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AU   Boldin M P; Mett I L; Varfolomeev E E; Chumakov I; Shemer-Avni Y;
     Camonis J H; Wallach D
TI   Self-association of the "death domains" of the p55   ***tumor***
       ***necrosis***     ***factor***   (  ***TNF***  ) receptor and
     Fas/APO1 prompts signaling for   ***TNF***   and Fas/APO1 effects.
SO   JOURNAL OF BIOLOGICAL CHEMISTRY, (1995 Jan 6) 270 (1) 387-91.
     Journal code: HIV. ISSN: 0021-9258.
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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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VOL. 270, NO. 1, Issue of January 6, pp. 191–196, 1995
Printed in U.S.A.

Self-association of the "Death Domains" of the p55 Tumor Necrosis Factor (TNF) Receptor and Fas/APO1 Prompts Signaling for TNF and Fas/APO1 Effects*

(Received for publication, October 4, 1994, and in revised form, November 4, 1994)

Mark P. Boldin‡, Igor L. Mett‡, Eugene E. Varfolomeev, Irina Chumakov, Yonat Sheinis-Avni, Jacques H. Camonis§, and David Wallach¶

From the Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel and §Denis Diderot University, INSERM Unit 248, 75010 Paris, France

Signaling by the p55 tumor necrosis factor (TNF) receptor and by the structurally related receptor Fas/APO1 is initiated by receptor clustering. Data presented here and in other recent studies (Wallach, D., Boldin, M., Varfolomeev, E. E., Sigua, Y., Camonis, H. J. and Mett, I. (1994) *Cytokine* 6, 556; Song, H. Y., Dunbar, J. D., and Donner, D. B. (1994) *J. Biol. Chem.* 269, 22492–22495) indicate that part of that region within the intracellular domains of the two receptors that is involved in signaling for cell death, as well as for some other effects (the "death domain", specifically self-associates. We demonstrate also the expected functional consequence of this association; a mere increase in p55 TNF receptor expression, or the expression just of its intracellular domain, is shown to trigger signaling for cytotoxicity as well as for interleukin 8 gene induction, while expression of the intracellular domain of Fas/APO1 potentiates the cytotoxicity of co-expressed p55 TNF receptor. These findings indicate that the p55 TNF and Fas/APO1 receptors play active roles in their own clustering and suggest the existence of cellular mechanisms that restrict the self-association of these receptors, thus preventing constitutive signaling.

Many cell surface receptors are triggered upon clustering. Unless restricted, this mode of triggering may result in their spontaneous signaling due to receptor chance encounters. The implications with regard to regulation of receptor function are underscored by the findings in the present study regarding the mechanisms of signaling by the p55 tumor necrosis factor (TNF)[1] receptor (p55 R) and Fas/APO1. These two structurally related receptors provide signals that can cause the death of cells expressing them, via structurally related sequence motifs in their intracellular domains (the "death domains", Refs. 2, 5, and 6). Dominant negative effects of mutations in these domains (2) and mimetic effects of antibodies against the two receptors (1, 7, 8) indicate that their signaling is initiated as a consequence of their clustering and self-interaction. TNF, and quite likely also the closely similar Fas ligand (9), occur as homotrimeric molecules (see, *e.g.*, Refs. 10 and 11) and thus can induce clustering of receptors merely by binding to them. Data presented here (see also Ref. 33) and in another recent study (4) show, however, that the intracellular domains of p55-R and of Fas/APO1 can aggregate even in the absence of their ligands, prompted by the ability of their death domains to self-associate. Additionally, we show that an increase in expression of these receptors, or even just of their death domain, can result in the induction of TNF and Fas/APO1-like effects, suggesting that the self-association of the death domain suffices to trigger signaling. These findings emphasize the need to elucidate how spontaneous signaling as a consequence of chance encounters between receptors normally is prevented.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen and Two-hybrid β-Galactosidase Expression Test—cDNA inserts were cloned by polymerase chain reaction, either from the full-length cDNAs cloned previously in our laboratory, or from purchased cDNA libraries. β-Galactosidase expression in yeasts (SFY526 reporter strain, Ref. 12) transformed with these cDNAs in the pGBT-9 and pGAD-GH vectors (DNA binding domain (DBD) and activation domain (AD) constructs, respectively) was assessed by a liquid test (13), as well as by a filter assay, which yielded qualitatively the same results (not shown). Two-hybrid screening (14) of a Gal4 AD-tagged HeLa cell cDNA library (Clontech, Palo Alto, CA) for proteins that bind to the intracellular domain of the p55-R (p55-IC), was performed using the *HF7c* yeast reporter strain. Positivity of the isolated clones was assessed by (*a*) prototrophy of the transformed yeasts for histidine when grown in the presence of 5 mM 3-aminotriazole, (*b*) β-galactosidase expression, and (*c*) specificity tests (interaction with SNF4 and lamin fused to Gal4 DBD).

In Vitro Self-association of Bacterially Produced p55-IC Fusion Proteins—Glutathione *S*-transferase (GST) and glutathione *S*-transferase-p55-IC fusion protein (GST-p55-IC) were produced as described elsewhere (15, 16). Maltose-binding protein (MBP) fusion proteins were obtained using the pMalRI vector (New England Biolabs) and purified on an amylose resin column. The interaction of the MBP and GST fusion proteins was investigated by incubating glutathione-agarose beads sequentially with the GST and MBP fusion proteins (5 μg of protein/20 μl of beads), first for 15 min and then for 2 h, at 4 °C. Incubation with MBP fusion proteins was carried out in a buffer solution containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 5% (v/v) glycerol or, when indicated, in that same buffer containing 0.4 M KCl or 5 mM EDTA instead of $MgCl_2$. Association of the MBP fusion proteins was assessed by SDS-polyacrylamide gel electrophoresis of the proteins associated with the glutathione-agarose beads, followed by Western blotting. The blots were probed with rabbit antiserum against MBP (produced in our laboratory) and with horseradish peroxidase-linked goat anti-rabbit immunoglobulin

* This work was supported in part by grants from Inter Lab Ltd. (Nes Ziona, Israel), from Ares Trading S.A. (Switzerland), and from the Israeli Ministry of Arts and Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Equivalent contributions were made by these two authors.

¶ To whom correspondence should be addressed. Tel.: 972-8-342941; Fax: 972-8-344165.

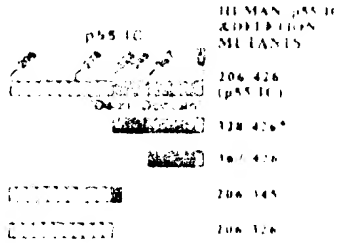
[1] The abbreviations used are: TNF, tumor necrosis factor; AD, activation domain; DBD, DNA binding domain; GST, glutathione *S*-transferase; IC, intracellular domain; IL-8, interleukin 8; MBP, maltose-binding protein; p55 IC, intracellular domain of the p55 R; p55 R, p55 tumor necrosis factor receptor; ELISA, enzyme linked immunosorbent assay.

TABLE 1

Self-association of the intracellular domains of p55 R and Fas/APO1 within transformed yeasts as assessed by a two-hybrid β-galactosidase expression test

Quantitative assessment of the interactions of Gal4 hybrid constructs encompassing the following proteins: the intracellular domain of human p55 R and the same deletion mutants; the cell death domains of mouse p55 R (residues 344–454), mouse Fas/APO1 (Fas IC, residues 169–306), human CD40 (CD40 IC, residues 216–277), and human p75 TNF receptor (p75 IC, residues 287–461, for residue numbering see Refs. 22, 23, and 30). SNF1 and SNF4 were used as positive (14) and lamin as a negative control (12). Proteins encoded by the Gal4 DBD constructs (pGBT9) are listed vertically, those encoded by the corresponding GAD constructs (pGAD GH) horizontally. The two deletion mutants denoted by asterisks were cloned in a two-hybrid screen of a HeLa cell cDNA library (Clontech, Palo Alto, CA) using p55 IC cloned in pGBT9 as "bait." Four of about 4×10^6 cDNA clones were positive, of which three were — upon re-correspondence to parts of human p55 R cDNA (two were identical, encoding residues 328–426, and one encoded residues 228–426). The fourth encoded an unknown protein. The β-galactosidase expression data are averages of assays of two independent transformants and are presented as amount of β-galactosidase product (1 unit of activity being defined as $OD_{420} \times 10^3$ divided by OD_{600} of the yeast culture and reaction time in minutes). The detection limit of the assay was 0.05 units. Variation between duplicate samples were in all cases less than 25% of the average. n.t. = not tested.

	HUMAN p55 IC & DELETION MUTANTS								OTHER PROTEINS					
	206-426 (p55 IC)	228-426*	328-426*	378-426	206-345	206-328	344-426	366-426	mouse p55 IC	mouse Fas IC	human CD40 IC	human p75 IC	SNF4	pGAD GH
HUMAN p55 IC & DELETION MUTANTS														
206-426 (p55 IC)	5.0	3.6	44.8	10.3	3.5	0	0	0						0
328-426*	5.7		61.8											0
378-426	0													
206-345	0		0											
206-328	0		0			0								
OTHER PROTEINS														
mouse p55 IC	0.17	0.18	3.0						2.8					0
mouse Fas IC	0	0	0.14						0	98.8	0	0		0
hu CD40 IC	0	0	0						0	0	0			0
human p75 IC	0								0		0			0
SNF1												2.7		0
Lamin	0	0	0						0					0
pUC19	0	0	0						0	0				



Induced Expression in HeLa Cells of the p55 R Fragments Thereof and Fas IC. HeLa cells expressing the tetracycline-controlled transactivator (the hxTA1 clone, Ref. 17) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.5 mg/ml neomycin. cDNA inserts encoding the p55 R or parts there of were introduced into a tetracycline-controlled expression vector (pUHD10–3, provided by H. Bujard). The cells were transfected with the expression construct (5 μg of DNA/6-cm plate) by the calcium phosphate precipitation method (16). Effects of transient expression of the transfected proteins were assessed at the indicated times after transfection in the presence or absence of tetracycline (1 μg/ml). Clones of cells stably transfected with the human p55 IC cDNA in the pUHD10–3 vector were established by transfecting the cDNA to hxTA 3 cells in the presence of tetracycline together with a plasmid conferring resistance to hygromycin, followed by selection for clones resistant to hygromycin (200 μg/ml). Expression of the cDNA was obtained by removal of tetracycline, which was otherwise maintained constantly in the cell growth medium.

Assessment of TNF Loc Effects Triggered by Induced Expression of the p55 R, Fragments Thereof or Fas IC. Effects of induced expression of the receptors and of TNF on cell viability were assessed by the neutral-red uptake method (18). Induction of interleukin 8 (IL-8) gene expression was assessed by Northern analysis. RNA was isolated using TRI Reagent (Molecular Research Center, Inc.), denatured in formaldehyde/formamide buffer, electrophoresed through an agarose/formaldehyde gel, and blotted to a GeneScreen Plus membrane (DuPont) in 10 × SSPE buffer, using standard techniques. Filters were hybridized with an IL-8 cDNA probe (19), nucleotides 1–392), radiolabeled by random primed DNA labeling kit (Boehringer, Mannheim, Germany), and washed stringently.

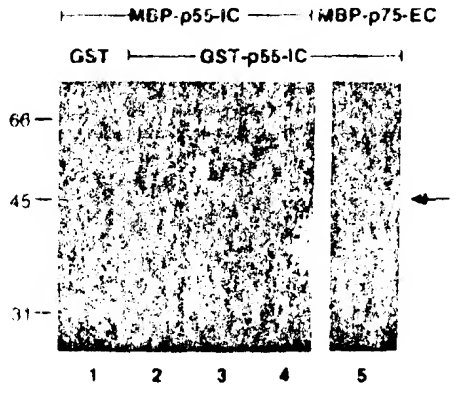


FIG. 1. **Self-association of the intracellular domain of p55-R *in vitro*: specific association of bacterially-produced fusion proteins containing the intracellular domain.** Interaction between fusion of human p55-IC to MBP (MBP-p55-IC) and to GST (*lane 2*) and the effect of EDTA (*lane 3*) and increased salt concentration (0.4 M KCl, *lane 4*) on this interaction. Interaction of MBP-p55-IC with GST (*lane 1*) and of GST-p55-IC with the fusion product of MBP and an irrelevant peptide (residues 195–229 in the mouse p75 TNF-R, MBP-p75-EC, *lane 5*, position indicated by an *arrow*) were also tested. SDS polyacrylamide gel electrophoresis (10% acrylamide) of the interacting proteins, followed by Western blotting, using anti-MBP antiserum, was performed as described under "Experimental Procedures."

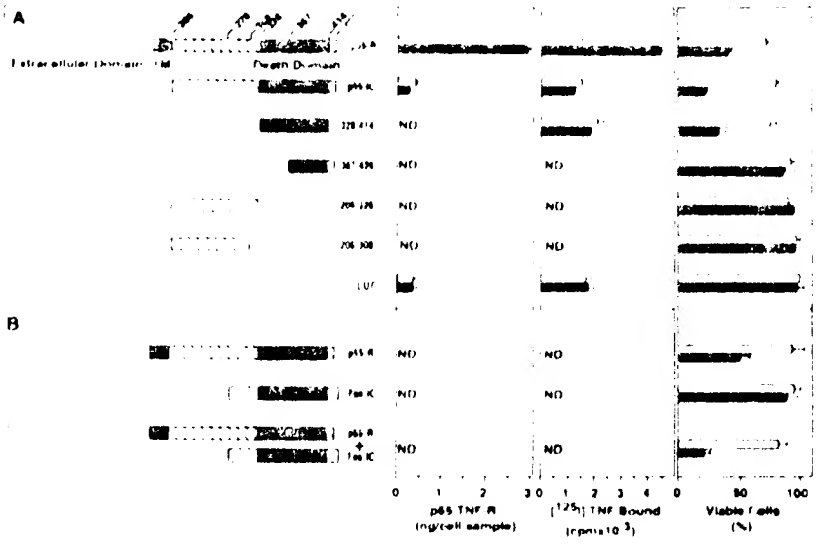


FIG. 2. **Ligand-independent triggering of a cytocidal effect in HeLa cells transfected with p55-R, or parts thereof, or with Fas-IC.** TNF receptor expression (*left*) and viability (*right*) in A, HeLa cells expressing, transiently, the full-length p55-R, p55-IC, or parts thereof, or, as a control, luciferase (*LUC*), and B, cells expressing Fas-IC, alone or together with the p55-R, using a tetracycline controlled expression vector. Cells transfected in the presence of tetracycline (1 μg/ml), which inhibits expression, ■. cells transfected in the absence of tetracycline. TNF receptor expression was assessed 24 h after transfection, both by ELISA, using antibodies against the receptor's extracellular domain (*middle*), and by determining the binding of radiolabeled TNF to the cells (*middle*). The cytocidal effect of the transfected proteins was assessed 48 h after transfection. Data shown are from one of three experiments with qualitatively similar results, in which each construct was tested in duplicate. *ND*, not determined.

Assessment of TNF Receptor Expression. TNF receptor expression in samples of 1×10^6 cells was assessed by measuring the binding of TNF, labeled with ^{125}I by the Chloramine T method, as described previously (20). It was also assessed by ELISA, performed as described for the quantification of the soluble TNF receptors (21), except for the use of radioimmune precipitation buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) to lyse the cells (50 μl/10^6 cells) and to dilute the tested samples. The soluble form of the p55-R, purified from urine, served as the standard.

RESULTS

Self-association of the death domain in the p55-R was observed by happenstance, on screening a HeLa cell cDNA library by the two-hybrid system technique (14) for proteins that bind to the intracellular domain of this receptor. Among the cDNAs whose products bound specifically to the intracellular domain-GAL4 DBD fusion-protein, several clones encoded parts of the p55-R intracellular domain (p55-IC, marked with asterisks in Table 1).

The extent of specificity in the self-association of p55-IC and the particular region involved was evaluated by the two-hybrid test. Table 1 shows the following: (*a*) The self-association of p55-IC is confined to a region within the death domain. Its N-terminus is located between residues 328 and 344, its C-terminus, close to residue 404, is somewhat upstream of the reported C-terminus of this domain (residue 414). (*b*) Deletion of the membrane-proximal part of p55-IC upstream of the death domain enhanced self-association, suggesting that this region has an inhibitory effect. (*c*) Mouse p55-IC self-associates and also associates with the death domain of human p55-R. (*d*) Examination of the self-association of the intracellular domains of three other receptors of the TNF/NGF receptor family: Fas/APO1, CD40 (22), and the p75 TNF receptor (23), showed that Fas-IC, which signals for cell death by a sequence motif related to the p55-R death domain, self-associates and associates to some extent with the p55-IC. However, CD40-IC, which provides growth stimulatory signals (even though also containing a sequence resembling the death domain), and p75-IC, which bears no structural resemblance to p55-IC, do not self-associate, nor do they bind p55-IC or Fas-IC.

An *in vitro* test of the interaction of a p55-IC GST bacterial fusion protein with a p55-IC MBP fusion protein confirmed that p55-R self-associates and ruled out involvement of yeast proteins (Fig. 1). The association was not affected by increased salt concentration or by EDTA (Fig. 1, *lanes 3* and *4*).

To evaluate the functional implications of the self-association of the death domain, we examined the way in which induced expression of p55-R, or of parts of it, affects cells sensitive to TNF cytotoxicity. Using an expression vector that permits strictly controlled expression of transfected cDNAs by a tetracycline regulated transactivator (17), we found that merely increasing p55-R expression in HeLa cells by expression of transiently transfected cDNA for the full-length receptor resulted in quite extensive cell death. Even greater cytotoxicity was observed when expressing just p55-IC (Figs. 2 and 3, *A* and *B*). Significant cytotoxicity was also observed when expressing just the death domain. In contrast, expression of parts of the p55-IC that lacked the death domain or contained only part of it (or expression of the luciferase gene, used as an irrelevant control) had no effect on cell viability. Expression of Fas-IC did not result in cytotoxicity, yet significantly enhanced the cytotoxicity of co-expressed p55-R (Fig. 2). The cytotoxicity of p55-IC was further confirmed using cells stably transfected with its cDNA; these cells continued to grow when p55-IC expression was not induced but died when p55-IC was expressed (Fig. 3*C*).

We examined also the effects of increased expression of p55-R and expression of just the intracellular domain of the receptor on the transcription of IL-8, known to be activated by TNF (19). As shown in Fig. 4, transfection of HeLa cells with a tetracycline-controlled construct encoding the p55-R cDNA induced IL-8 transcription. An even stronger induction was observed in cells transfected with the cDNA for p55-IC. In both cases, the induction occurred only when tetracycline was excluded from the cell growth medium, indicating that it occurs as a consequence of expression of the transfected p55-R or p55-IC. Transfection with luciferase cDNA, as a control, had no effect on IL-8 transcription.

DISCUSSION

Studies employing the two-hybrid technique suggested that the intracellular domain of the p55-R self-associates and located this self-association to a part of a region found to be critical for signaling by this receptor (Ref. 4 and the present report, see also Ref. 33). Further tests confirmed that this association is not artifactual, as may well occur in the yeast genetic test (24), and indicated that it has functional conse-

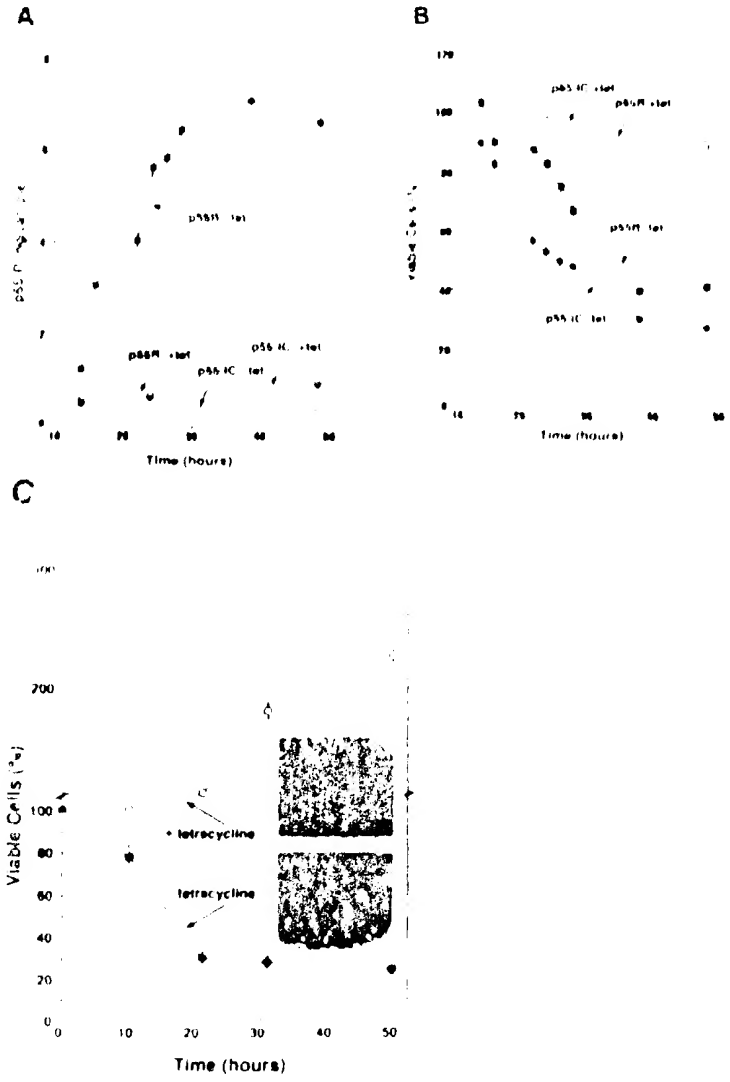


FIG. 3. **Ligand-independent triggering of a cytocidal effect in HeLa cells transfected with p55-R or its intracellular domain: kinetic study of transient expression of the receptor and its expression in a stable transfectant.** *A*, TNF receptor expression assessed by ELISA; *B*, cell viability, in transient transfection of the full length receptor (■) and of p55 IC (●) in the presence or absence of tetracycline (empty and solid notes, respectively), assessed at various times after incubation with the transfected DNA. *C*, effect of p55 IC expression on the viability of cells transfected stably with the cDNA, assessed at various times after replacement of the cell growth medium with fresh medium either with or without tetracycline. Photographs were taken 36 h after tetracycline removal.

quences. The self association could be shown to occur also *in vitro*, using GST and MBP p55 IC fusion proteins, thus ruling out involvement of yeast proteins or of the Gal4 DBD or AD in this association. Moreover, the expected functional consequence of this association could be demonstrated, namely occurrence of spontaneous signaling under conditions that permit receptor aggregation. A mere increase in p55-R expression, or even expression just of the intracellular domain of the receptor or of its death domain, was found to be sufficient to trigger signaling for cytotoxicity as well for expression of the TNF-inducible *IL-8* gene within cells.

Normally, cells expressing the p55-R do not exhibit TNF effects unless exposed to this cytokine. Presumably, cells possess some mechanisms that reduce the self-association of the receptor and impose on it ligand dependence. Probably self-association of the receptors is in part restricted by mechanisms that maintain their self-surface expression at a low level. It may also be restricted by constraints imposed on the death domain in the receptor by other regions in the p55-R molecule. To some extent, self association of the death domain seems to be inhibited by the membrane-proximal part of the intracellular domain (Table I). Crystallographic studies of the extracel-

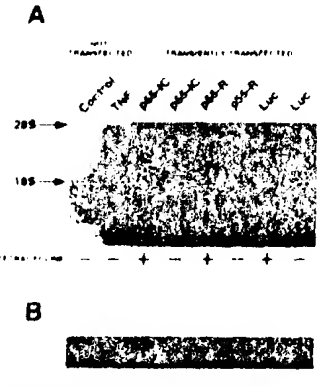


FIG. 4. **Ligand-independent induction of IL-8 gene expression in HeLa cells transfected with p55-R or its intracellular domain.** *A*, Northern analysis of RNA (7 μg/lane), extracted from HeLa (HTa-1) cells, untreated or treated with TNF (500 units/ml) for 4 h, autoradiography performed for 6 h; or the HTa-1 cells 24 h after their transfection (in the presence or absence of tetracycline) with p55-IC, the p55-R or luciferase cDNA (autoradiography for 18 h). *B*, methylene blue staining of 18 S rRNA. For other details, see "Experimental Procedures."

final domain of the receptor suggest that also this domain mediates an inhibitory effect. They indicate that, in the absence of TNF, the extracellular domains of neighboring p55 R molecules are capable of interacting in a way that obviates association of their intracellular domains.[7] Such interaction may well prevent spontaneous signaling by the receptors and allow their intracellular domains to self associate only after TNF binding.

The intracellular domain of Fas/APO1, which bears marked structural similarity to that of the p55 R and that likewise signals for cell death, was found also to self associate and thus trigger signaling, suggesting that this receptor, too, plays an active role in its aggregation and is subject to control mechanisms that antagonize its propensity to self associate. This may well be the case also for a number of other receptors, for example several tyrosine kinase receptors, including Neu/HER-2 and the epidermal growth factor receptor, that are found, just like the p55 R, to signal spontaneously when expressed at high levels as well as after deletion of their extracellular domain (see, *e.g.*, Refs. 25–27, and references therein).

Interestingly, the p75 TNF receptor, even though it has, like p55 R and Fas/APO, the ability to signal for cell death (28), does not display self association, nor does a high level of expression of this receptor result in spontaneous signaling (29). Apparently, the mode of signaling for cell death by this receptor differs from that of the p55 R (29).

Most likely, the self associations of p55 R and Fas/APO1 serve to fortify the aggregated state imposed on them by their ligands. Such a mechanism has certain functional advantages. It may augment signaling and also provide ways for modulation of signaling by mechanisms that act within the cell. An intriguing possibility for such modulation is indicated by the slight association between p55-IC and Fas-IC, which may allow cross talk between the two cell death inducing receptors (7).

The propensity of these receptors to self-associate may permit also a kind of derangement of regulation that would not be expected if their aggregation occurred in a passive manner. It can lead to spontaneous signaling, independent of the ligand, in situations in which the mechanisms restricting the self-association of the receptors fail to function properly. Such ligand-independent function is, in the case of growth factor receptors, a well known cause for the uncontrolled growth of malignant cells. In receptors that signal for cytotoxicity, it may contribute to uncalled-for death of cells, as observed, for example, in response to cytopathic viruses and various other pathogens.

[7] J. Naismith and S. Sprang, personal communication.

Acknowledgments. We are grateful to Drs. Herman Bujard and Sabina Freundlieb for providing us with the reagents for tetracycline-controlled expression and to Ada Inbeman for careful handling of the cultured cells.

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AU   Brakebusch C; Varfolomeev E E; Batkin M; Wallach D
TI   Structural requirements for inducible shedding of the p55
       ***tumor***     ***necrosis***     ***factor***   receptor.
SO   JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 Dec 23) 269 (51) 32488-96.
     Journal code: HIV. ISSN: 0021-9258.
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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 269, No. 51, Issue of December 23, pp. 32488–32496, 1994
Printed in U.S.A.

Structural Requirements for Inducible Shedding of the p55 Tumor Necrosis Factor Receptor*

(Received for publication, August 19, 1994, and in revised form, October 12, 1994)

Cord Brakebusch‡, Eugene E. Varfolomeev§, Michael Batkin, and David Wallach¶

From the Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot, Israel, 76100

Induced shedding of the p55 tumor necrosis factor receptor (p55-R) was previously shown to be independent of the amino acid sequence properties of the intracellular domain of this receptor. We now find it also independent of the sequence properties of the transmembrane domain and of the cysteine-rich region that constitutes most of the extracellular domain of the receptor. The shedding is shown to depend solely on the sequence properties of a small region within the spacer that links the cysteine-rich region in the extracellular domain to the transmembrane domain. Detailed tests of effects of mutations in the spacer on the shedding indicate that the process is independent of the amino acid side-chain identity in this region except for a limited dependence on the identity of 1 residue (Val-173), located downstream to the putative major cleavage site of the receptor. It is strongly affected, however, by some mutations that seem to change the conformation of the spacer region. These findings suggest that a short amino acid sequence in the p55-R is essential and sufficient for its shedding and that the shedding is mediated either by a protease with limited sequence specificity or by several different proteases that recognize different amino acid sequences, yet it strictly depends on some conformational features of the cleavage region in the receptor.

Many cell-surface proteins occur also in soluble forms (see Refs. 1 and 2 for review). Some of these soluble proteins are produced independently of their corresponding cell-surface forms. They are translated from distinct transcripts, formed by alternative splicing mechanisms from the same genes as those encoding the cell-surface proteins. Soluble forms of other cell-surface proteins are proteolytically derived from the corresponding cell-surface molecules. Whereas there is quite detailed knowledge of mechanisms of the former kind (3, 4), little is known of the proteolytic mechanisms by which cell-surface proteins are shed. None of the proteases contributing to the shedding has been identified so far, nor is there any knowledge of the way in which these proteolytic activities are regulated.

In the present study, we explored the mechanisms of formation of the soluble form of the p55 tumor necrosis factor receptor (TNF-R).[1] Both this receptor and the other molecular species of TNF-R (the p75-R) are single transmembrane domain proteins, in which the extracellular domains bind TNF and the intracellular domains provide signals for the TNF effects (5–12). The TNF-binding regions in the extracellular domains of the two receptors are similar. They are comprised of a conserved cysteine-rich sequence, found also in the extracellular domains of several other, evolutionarily related receptors (the TNF/nerve growth factor receptor family; (13)). In the p55-R, this cysteine-rich module is located at a rather short distance from the transmembrane domain (16 amino acid residues), while in the p75-R, it is linked to the transmembrane domain by a rather long spacer region (of 56 amino acid residues).

Soluble forms of the p55 and the p75 TNF-Rs, whose structures correspond to those of the TNF-binding regions in the corresponding cell-surface receptors, occur in various body fluids (6, 14). They can be also discerned in the growth medium of various cultured cells (10, 12, 15, 16). These soluble receptors bind TNF in a reversible manner and consequently are capable of serving as "buffering" agents for this cytokine; they restrict the availability of TNF to the cell-surface TNF-Rs yet can also extend its functional duration by stabilizing the TNF molecules to which they bind (17). Formation of the soluble TNF-Rs is increased in various disease states, as well as after TNF injection, allowing these proteins to function as feedback regulators of TNF activity (14, 18). Induced formation of the soluble TNF-Rs can also be observed in cell culture. A variety of cells produce the soluble TNF receptors when treated with 4β-phorbol 12-myristate 13-acetate (PMA), a well known activator of protein kinase C (15, 16, 19). Some cells have been found to produce soluble TNF receptors in response to cell-specific biological stimuli. Granulocytes, for example, produce the soluble receptors when treated with the chemotactic peptide formyl-Met-Leu-Phe (20), and T cells produce them in response to T cell mitogens (21).

When effectively induced, formation of the soluble TNF-Rs is accompanied by a marked decrease in the amount of cell-surface TNF-Rs (19, 22, 23). This phenomenon, the rapidity of induction, and the fact that soluble TNF-Rs can be generated by cells transfected with the cDNA for the cell-surface receptor, thus excluding the possibility of alternative splicing (10, 16), indicate that the soluble TNF-Rs are formed primarily, if not solely, by proteolytic cleavage of the cell-surface receptors. Sequence analysis of soluble TNF-Rs isolated from human urine suggested that the cell-surface TNF-Rs are cleaved in the spacer region between the cysteine-rich TNF-binding domain and the transmembrane region. Two different C termini were found for the soluble p55-R isolated from urine. The major C terminus is

* This work was supported in part by grants from Inter-Lab Ltd., Ness-Ziona, Israel, and Ares Trading S. A., Switzerland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Portions of this work (performed during a stay at the Weizmann Institute) have been submitted as part of the requirements for the Ph.D. degree from the University of Hannover, Germany. Present address: Dept. of Molecular Biology, Max-Planck-Institute, Martinsried bei München, Germany.

§ A recipient of the International Cancer Technology Transfer award from the International Union Against Cancer, Geneva, Switzerland.

¶ To whom correspondence should be addressed. Tel.: 972-8-343941; Fax: 972-8-343165.

[1] The abbreviations used are: TNF-R, TNF receptor; EGF-R, epidermal growth factor receptor; hu-p55-TNF-R, human p55 TNF receptor; p55-R, p55 TNF receptor; p75-R, p75 TNF receptor; PMA, 4β-phorbol 12-myristate 13-acetate; PV, pervanadate; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium.

Asn-172, 6 amino acids downstream from the C-terminal cysteine in the extracellular domain (Asn-172) (10). A minor fraction of the protein is longer by 2 residues, having Lys-174 as a C terminus (24). The major C terminus of the soluble p75-R isolated from urine corresponds to Val-192, located 14 amino acids downstream from the C-terminal cysteine.[2]

To investigate the shedding mechanism of the p55-R, we examined the nature of the structural specificity in this proteolytic process. In an attempt to define the relationship between the structural requirements for the shedding and for the signaling activity of the receptor, we examined the effects of cytoplasmic deletion on these two activities. C-terminal truncation of the receptor by more than 21 residues prevented signaling for the cytocidal effect of TNF. However, deletion of the intracellular domain, even when practically complete, did not interfere with either spontaneous or PMA-induced shedding of the receptor (19, 25). In contrast, receptor mutants lacking the spacer region were not shed while still having the ability to trigger the cytocidal effect (25). In the present study, we examine the shedding of chimeric molecules in which various parts of the p55-R were replaced with corresponding regions in the EGF receptor (EGF-R), a receptor that is poorly shed. Our analysis indicated that the spacer region in the p55-TNF-R is essential and sufficient for its shedding. Further examination of effects of mutations within the spacer on shedding suggested that the proteolytic cleavage of the receptor is affected only to a small extent by the nature of the side chains of the amino acid residues in this region but is strongly dependent on the conformation of the spacer region.

EXPERIMENTAL PROCEDURES

Construction of p55-TNF-R Mutants and p55-R-EGF-R Chimeras—The cDNA of the hu-p55-TNF-R (10) was digested with *Bam*II and *Nhe*I, resulting in removal of most of the 5'- and 3'-non-coding sequences. The p55-TNF-R mutants were generated by oligonucleotide-directed mutation, using the Altered Sites mutagenesis kit (Promega). The mutations were confirmed by sequencing the regions of interest. Fragments of the hu-p55-TNF-R and of the EGF-R cDNAs used for creation of receptor chimeras were produced by polymerase chain reaction, using the Vent DNA polymerase (New England Biolabs). For reasons of availability at different stages of the study, some of the chimeras (employed in the experiments presented in Figs. 1 and 3*B*) were constructed using mouse EGF-R cDNA (26), kindly provided by Dr. D. Givol of the Weizmann Institute (Rehovot, Israel) and others (employed in the experiment presented in Fig. 3*C*) using human EGF-R cDNA (27), kindly provided by Drs. G. Merlino and I. Pastan (National Institutes of Health). The various chimeras were constructed as follows: construct 2, residues −28–181 of the hu-p55-TNF-R were linked to residues 623–654 of the mouse EGF-R; construct 3, residues −28–168 of the hu-p55-TNF-R were linked to residues 611–654 of the mouse EGF-R; construct 4, residues −28–168 of the hu-p55-TNF-R were linked to residues 623–654 of the mouse EGF-R; construct 5, residues −28–181 of the hu-p55-TNF-R were linked to residues 611–654 of the mouse EGF-R; construct 6, residues −24–653 of the human EGF-R; construct 7, residues −24–611 of the human EGF-R were linked to residues 170–181 of the hu-p55-TNF-R, and those were linked to residues 621–653 of the human EGF-R. To facilitate cloning of the fused receptor fragments, restriction sites were introduced into their nucleotide sequences; consequently, Gly-611 and Cys-612 in the EGF-R were replaced in constructs 3 and 5 with serine and phenylalanine residues, respectively, Ile-624 in the EGF-R in constructs 2 and 4 was replaced with a phenylalanine residue, and Glu-610, Gly-611, and Ile-622 in the EGF-R in construct 7 were replaced with glycine, serine, and phenylalanine residues, respectively. For constitutive expression of the wild-type or mutated receptors in A9 cells, they were introduced into the eukaryotic expression vector pMPSVEH (28), kindly provided by Dr. H. Hauser (Gesellschaft für Bitechnologische Forschung mbH, Braunschweig, Federal Republic of Germany), which contains the myeloproliferative sarcoma virus promoter. For transient expression of the receptors in COS-7 cells, they were introduced into the pEXV1 vector (29), which contains the SV40 virus enhancer and early promoter. In all of the hu-p55-TNF-R constructs expressed in COS-7 cells, the receptor was cytoplasmically truncated from residue 207 downstream (in addition to the other specified mutations).

Constitutive and Transient Expression of the Wild-type and Mutant Receptors—A9 (30) and COS-7 (31) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (growth medium). The A9 cells were transfected with pMPSVEH expression constructs together with the pSV2neo plasmid, and cell colonies constitutively expressing these constructs were isolated as previously described (19). Transient expression of pEXV1 constructs in COS-7 cells was as described earlier (32). Briefly, 1 day after the COS-7 cells were seeded at 60% cell density, they were transfected by applying the DNA of the constructs to them for 4 h at a concentration of 3 μg/ml in DMEM (4 ml/10-cm dish, 10 ml/15-cm dish) containing DEAE dextran (200 μg/ml, Pharmacia, Uppsala, Sweden). The cells were then rinsed with DMEM and incubated for 2 min in phosphate-buffered saline (0.154 M sodium chloride plus 10 mM sodium phosphate, pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10% (v/v) dimethyl sulfoxide. The cells were rinsed and further incubated for 10 h in growth medium and then detached by trypsinization and seeded either into 1.5-cm culture plates (10^5 cells/plate) or (to assess the shedding of metabolically labeled EGF-R) into 15-cm plates (1.2×10^6 cells/plate). Expression and efficacy of shedding of receptors encoded by the transfected constructs were assessed 48 h later.

Determination of Binding of TNF and EGF to Cells—Recombinant human TNF-α (6×10^7 units/mg protein) (Genentech Co., San Francisco), kindly provided by Dr. G. Adolf of the Boehringer Institute (Vienna, Austria), was radiolabeled with chloramine T to a specific radioactivity of 500 Ci/mmol (33). EGF (β-urogastrone, Boehringer Mannheim) was labeled with ^{125}I to a specific radioactivity of 300 Ci/mmol, using the IODO-GEN reagent (Pierce), following the instructions of the producer. Binding of radiolabeled TNF and EGF to cells was determined by applying them to the cells on ice at a concentration of 1 nM, either alone or with a 100-fold excess of the unlabeled cytokines, as previously described (19).

Measurement of the Shedding of the Soluble Forms of hu-p55-TNF-R and EGF-R—A9 cells constitutively expressing the transfected constructs were seeded 24 h prior to the assay into 1.5-cm tissue culture plates at a density of 2.5×10^5 cells/plate. COS-7 cells expressing transiently transfected constructs were seeded into 1.5-cm tissue culture plates 48 h prior to the assay as described above. At time 0, some of the plates were placed on ice to determine the binding of radiolabeled TNF or EGF to the cells prior to induction of shedding. The medium in the other plates was replaced with fresh DMEM (200 μl/plate) either without serum (for tests in which PV was the agent used to induce shedding) or with 10% fetal calf serum (for the other tests). Unless otherwise indicated, PMA (20 ng/ml) or PV (100 μM, prepared as described in Ref. 34) was applied to the cells for 1 h. Application of chloroquine (50 μg/ml), ammonium chloride (10 mM), or cycloheximide (50 μg/ml) to the cells was done 30 min prior to application of PMA or PV, followed by further incubation with these agents for 20 min after addition of the latter reagents. Upon termination of incubation with the shedding inducing agents, the plates were transferred to ice to determine the binding of radiolabeled TNF or EGF to the cells. The amounts of the soluble form of the hu-p55-TNF-R in the cell growth media were determined after centrifugation at $3000 \times g$ for 5 min to remove detached cells and cell debris, followed by 5-fold concentration of the media using the SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, NY). The determination was performed by two-site capture enzyme-linked immunosorbent assay using a mouse monoclonal antibody and rabbit antiserum against this protein as described (35).

To assess the formation of the soluble form of the EGF-R, COS-7 cells transfected with the EGF-R constructs (1.2×10^6 cells, seeded into 15-cm dishes as described above) were labeled with [^{35}S]methionine by incubation for 10 h at 37 °C in DMEM (methionine-free) containing 70 μCi/ml [^{35}S]methionine and 2% dialyzed fetal calf serum. The cells were then rinsed and further incubated for 1 h in growth medium containing PMA (20 ng/ml). The medium was collected, cleared of cell debris by spinning, and then further cleared of proteins that nonspecifically bind to protein A by incubating it twice at 4 °C for 4 h with immobilized protein A (100 μl/7 ml medium/plate, Repligen Corp., Cambridge, MA), once alone and once in the presence of 10 μg of irrelevant mouse monoclonal antibodies. Immunoprecipitation was then performed by incubation of the medium samples at 4 °C for 2.5 h with a monoclonal antibody against the human EGF-R (528 (Ref. 36); the same immunoprecipitation pattern was observed when anti-EGF-R monoclonal antibody 108 (Ref. 37) was used) or, as a control, with a monoclonal antibody against

[2] S. Villa, U. Bucciarelli, and D. Wallach, unpublished data.

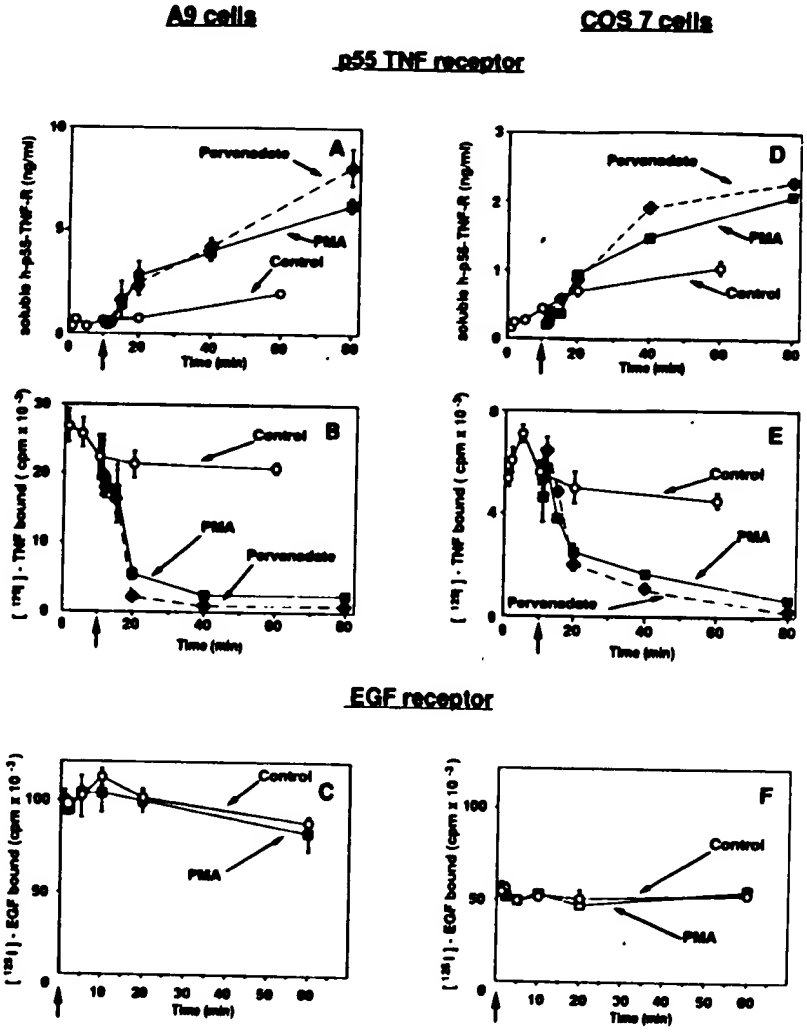


FIG. 1. **Kinetics of the effects of PMA and PV on formation of the soluble form of the hu-p55-TNF-R (*A* and *D*) and on expression of the cell-surface hu-p55-TNF-R (*B* and *E*) and cell-surface mouse EGF-R (*C* and *F*) in clones of A9 cells that constitutively express them (*left panels*) and in COS-7 cells that transiently express them (*right panels*).** ○, spontaneous shedding; ■, shedding induced by PMA (20 nM); ◆, shedding induced by PV (100 μM). Both receptors were cytoplasmically truncated (hu-p55-TNF-R, residues –28–206; mouse EGF-R, residues –24–654). A9 cells expressing the full-length receptor exhibited similar kinetics of shedding (19). PMA and PV were applied in *A*, *B*, *D*, and *E* 10 min after the beginning of the test and in *C* and *F* at time 0 (*arrow*). Cell-surface binding of radiolabeled TNF and EGF and concentrations of the soluble hu-p55-TNF-R in the cell growth media were determined at the indicated time points as described under "Experimental Procedures."

the hu-p55-TNF-R (29 (Ref. 38)), each at 5 μg/sample, followed by further incubation for 2.5 h with immobilized protein A (40 μl). The protein A beads were washed three times with phosphate-buffered saline containing 0.2% sodium deoxycholate and 0.2% Nonidet P-40, and the proteins bound to them were then analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions (7.5% acrylamide). Autoradiography was performed after treatment of the gel with the Amplify intensifying reagent (Amersham International plc, Amersham, UK).

Presentation of the Data—All data on receptor shedding presented in this paper are representative examples of at least four experiments with qualitatively similar results, in which each construct was tested in triplicate. As explained under "Results," the efficacy of construct expression varied rather extensively (in their constitutive expression among different cell clones and in their transient expression among different constructs). The data on the extent of shedding have therefore been normalized by relating them to the initial receptor levels in the cells prior to the induction of shedding. The amounts of receptors obtained after induction are presented as percentages of the their initial amounts, and the amounts of soluble receptors formed as a consequence of the shedding are presented in relative units, *i.e.* amount of soluble receptors produced during shedding induction (in pg) per amount of cell-surface receptors just prior to shedding induction (in cpm × 10^{-3} of cell-bound radiolabeled TNF). To save space, since in no case did this normalization affect the qualitative character of the data, only an example of the data prior to normalization is presented (Fig. 2). Likewise, just an example of the data of the spontaneous shedding is presented in Fig. 2, as in all constructs tested, the efficacy of spontaneous formation of soluble receptors was proportional to the efficacy of shedding induced by PMA or PV. Residue numbering in the hu-p55-TNF-R is according to Ref. 9, in the mouse EGF-R according to Ref. 26, and in the human EGF-R according to Ref. 39.

RESULTS

General Features of the Test Systems

Effects of mutations in the hu-p55-TNF-R on its shedding were assessed by employing two ways of receptor expression. Initially, mutation effects were assessed by constitutively expressing the mutants in the mouse A9 cells, which are highly sensitive to the cytocidal effect of TNF. This allowed us to determine the relationship between the structural requirements for shedding and for signaling for the cytocidal effect (19, 25). Later on, having found that the shedding and the signaling involve different molecular structures, we employed a more rapid and less laborious way of assessment by transient expression of the mutants in the monkey COS-7 cells. Because the intracellular domain is not necessary for the shedding (19, 25), an intracellularly truncated form of the p55-R, which could be expressed more efficiently than the full-length receptor, was used in the transient expression tests. Similarity between the shedding characteristics of mutants expressed in the A9 cells and (with the additional C-terminal deletion) in COS-7 cells confirmed the equivalence of the two test systems (see Figs. 1, 3, 5, and 6).

In both the A9 and the COS-7 cells, PMA, known to activate protein kinase C (40), and pervanadate (PV), which augments tyrosine phosphorylation (34), induced effective shedding of transfected hu-p55-TNF-R. This induction was manifested in decreased TNF binding to the cells concomitantly with en-

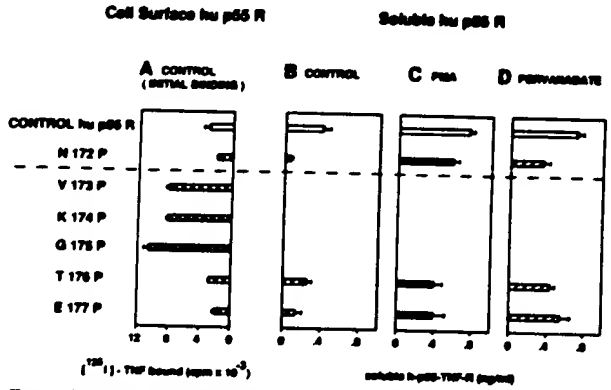


FIG. 2. **Inverse relationship between shedding efficacy of the various hu-p55-TNF-R proline-replacement mutants and their cell-surface expression in transiently transfected COS-7 cells.** *A*, binding of ^{125}I-labeled TNF to cells of the transfected cultures just prior to the induction of shedding. *B–D*, amounts of the soluble hu-p55-TNF-R accumulated within 1 h in control untreated cultures, in cultures treated with PMA (20 nM), and in cultures treated with PV (100 μM), respectively.

hanced accumulation of soluble p55-R in the cell growth media (Fig. 1, *A*, *B*, *D*, and *E*). The small amount of binding of TNF to the endogenous receptors of nontransfected cells was also eliminated by PMA or pervanadate treatment, apparently because of shedding of these endogenous receptors (data not shown). The induced shedding of both the transfected and the endogenous receptors was rapid and could not be inhibited with cycloheximide but was arrested at 4 °C, indicating that it involves mechanisms that are energy-dependent but independent of new protein synthesis. It was not inhibited by ammonium chloride or by chloroquine, both inhibitors of lysosomal functions (data not shown). The rate of spontaneous formation of soluble p55-R in the absence of PMA or PV was higher in the COS-7 cells than in the A9 cells. In addition, the COS-7 cells differed from the A9 cells in displaying an initial transient increase of TNF binding, apparently triggered by the growth-medium replacement at the beginning of the test (Fig. 1*E*).

The level of the transfected receptors in the A9 stable transformants varied significantly from one cell clone to another. Accordingly, the amounts of soluble receptors produced by the cells and their eventual cell-surface levels after shedding induction also varied in proportion to the amount of cell-surface receptors initially expressed by the particular clone. The transient transfection tests, in which shedding was assessed using the whole transfected culture, were not affected by such interclonal variation. They did, however, display variation of another kind related to the nature of the mutants. As exemplified in Fig. 2, which presents the p55-R levels in cells expressing various proline-replacement mutants of the receptor, the amounts of the receptors were inversely correlated with their efficacy of shedding. Apparently, this inverse correlation reflects an impact of the spontaneous shedding of the receptors, which was proportional to their rate of induced formation (compare Fig. 2, *C* and *D* with *A* and *B*) on their steady-state cell-surface level. To account for this intermutant variation and for the interclonal variation observed in the A9 stable transformants, we normalized the shedding data by relating them to the amounts of receptors in the untreated cells (see "Experimental Procedures"). Since the amounts of transfected receptors were at least 10-fold higher than the amounts of endogenous receptors, the contribution of the latter to TNF binding was ignored in this calculation.

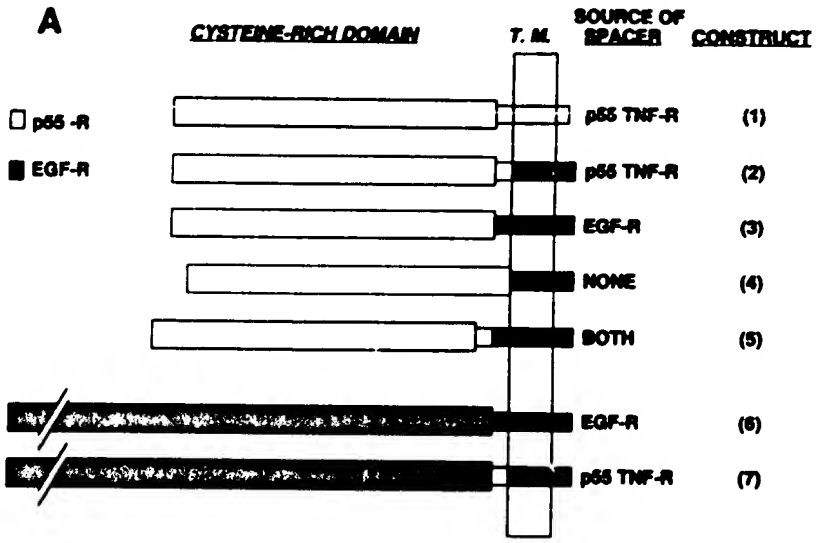
Use of Chimeras of the p55-R and the EGF-R to Assess the Involvement of the Major Domains of the TNF-R in Its Shedding

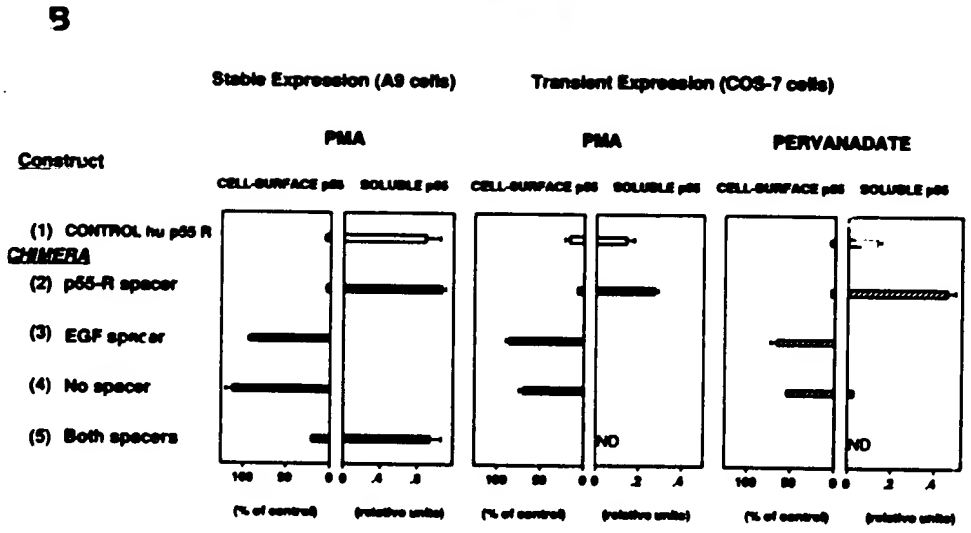
The intracellular domain of the p55-R apparently does not participate in its shedding (19). To explore the involvement of the transmembrane domain in the shedding, we first created a series of mutants of the p55-R in which individual amino acid residues in the transmembrane domain were replaced with others (Pro-186 with alanine, Cys-194 with alanine, serine, or tyrosine, Ser-196 with alanine, threonine, cysteine, tyrosine, or phenylalanine, and Tyr-204 with phenylalanine) and examined the shedding of these mutants after constitutively expressing them in A9 cells. All the mutants were found to shed normally (data not shown).

To obtain a more comprehensive view of the involvement of the sequence properties of the transmembrane domain and of the other major receptor domains in the shedding, we attempted to identify a receptor that is resistant to the shedding mechanism and to employ this receptor for exploring the substrate specificity in this mechanism. PMA treatment of cells expressing the EGF-R has been shown to result in decreased signaling and high affinity EGF binding by this receptor. However, in contrast to the effect of PMA on the p55-R, it does not affect the overall binding of EGF by cells, suggesting that its effects on the EGF-R do not reflect induced receptor shedding (Refs. 41 and 42 and Fig. 1, *C* and *F*). We therefore examined the way in which the shedding of the p55-R is affected by replacement of its various regions with corresponding regions in the EGF-R. Since the PMA-induced modulation of the function of the EGF-R seems to be at least partly due to PMA-induced phosphorylation of its intracellular domain, we introduced a truncated cytoplasmic domain of the receptor lacking potential protein kinase C phosphorylation sites into these chimeras.

The various chimeras differed markedly in efficacy f shedding (Fig. 3). (i) Replacement of the intracellular and transmembrane domains of the p55-R with the corresponding regions in the truncated EGF-R did not prevent spontaneous or induced (PMA, PV) shedding (construct 2). (ii) Further replacement of the spacer region of the p55-R (amino acids 169–181) by an equally long membrane-proximal portion of th EGF-R spacer almost completely eliminated cleavage of the chimeras (construct 3), as did also deletion of the spacer (construct 4). (iii) A chimera containing both the spacer of the p55-TNF-R and an equal-sized portion of the EGF-R displayed effective shedding (construct 5; this particular chimera was very poorly expressed in transiently transfected COS-7 cells and could therefore be tested only when constitutively expressed in the A9 cells). (iv) Using monoclonal antibodies against the extracellular domain of the EGF-R, we did detect small amounts of a soluble form of the EGF-R in the supernatant of cells transfected by the cDNA of this receptor (see legend to Fig. 3). However, production of this soluble form was not affected by PMA. Moreover, PMA did not cause any decrease in cell-surface expression of the EGF-R, indicating that the intracellularly truncated EGF-R is indeed resistant to the shedding mechanism activated by PMA (construct 6, Fig. 3*C*). (v) Replacement of the 11 membrane-proximal amino acids of the truncated EGF-R with the spacer of the p55-R markedly enhanced the formation of soluble EGF-R. Moreover, shedding of the EGF-R chimera that contained the p55-R spacer was strongly enhanced by PMA, as reflected both in enhanced formation of the soluble form of this receptor and in an observed decrease in expression of the cell surface EGF-R (construct 7, Fig. 3*C*).

Taken together, these findings indicate that the efficacy of shedding f the p55-TNF-R depends on some specific sequence properties of the spacer region in its extracellular domain but is





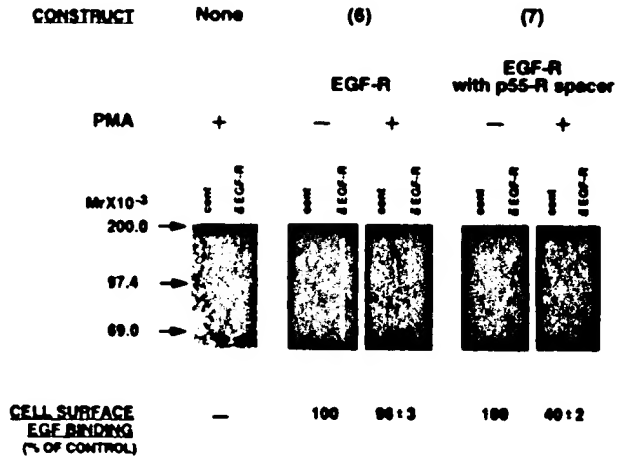


FIG. 3. **Shedding of various chimeras of p55-R and EGF-R.** Schematic presentation (*A*) and shedding data (*B* and *C*) of the chimeras is shown. *B*, chimeras whose cytokine-binding domain was derived from the p55-R. *1*, control hu-p55-TNF-R; *2*, the cytokine-binding and spacer regions of the TNF-R linked to the transmembrane and intracellular domains of the EGF-R; *3*, the cytokine-binding region of the TNF-R linked

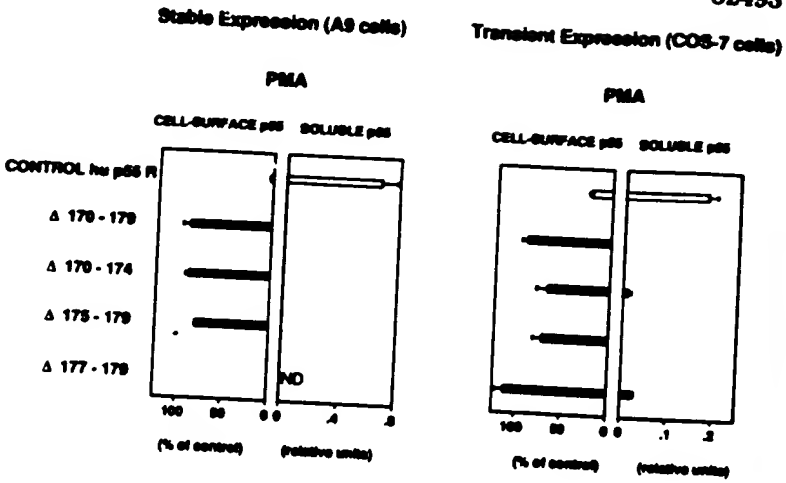


FIG. 4. **Effects of deletions of 3-, 5-, or 10-amino acid portions of the hu-p55-TNF-R spacer on its shedding (assessed after treatment for 1 h with PMA (20 nM) or PV (100 μM)).**

hardly affected by the sequence properties of any other region in the receptor.

Mutational Study of the Spacer Region

Effects of Amino Acid Deletions—To determine what particular sequence properties of the spacer region influence the shedding, we first examined how the shedding is affected by deletion of various parts of this region. As noted before (25), deletion of residues 170–179 prevented the shedding. So did deletion of 5-residue portions of the spacer, either at the region presumed to be the site of cleavage of the receptor (residues 170–174) or downstream from it (residues 175–179) (Ref. 25 and Fig. 4). Deletion of a 3-residue portion at this downstream region also decreased the shedding to some extent (residues 177–179) (Fig. 4).

When we examined the effect of deletion of various pairs of adjacent amino acid residues within the spacer region, we found that shedding was markedly decreased upon deletion of residues 172 + 173 or 173 + 174. Deletion of Val-173 alone also reduced the shedding, although to a lesser extent than its deletion together with either of its adjacent residues. The shedding was hardly affected, however, by deletion of any other amino acid pair in the spacer region (Fig. 5).

Effects of Amino Acid Replacements—To determine whether the observed effects of deletion reflect a dependence of the shedding mechanism on any particular residue within the spacer, we examined the way in which the shedding is affected by replacement of various residues in this region. Because alanine residues fit well in many secondary structures, substitution with alanine is the method of choice for testing the functional significance of a residue with minimal distortion of the overall protein structure (43). "Alanine-scanning mutagenesis" of the region between residues 171 and 181 failed to reveal any residue whose identity is of importance for the shedding (Fig. 6). In view of a prior report suggesting that simultaneous changes at the sites of residues 173 and 174 may have a greater effect on the shedding than alteration of either of these residues alone (44), we also examined a receptor mutant in which both of these residues were replaced with alanine. This mutant, like the others, was found to shed normally (Fig. 6).

In view of the observed decrease in shedding upon deletion of Val-173 and the additional decrease on its deletion together with Asn-172 or Lys-174, we further examined the effect of amino acid replacements at these sites. Replacement of Asn-172 with glutamic acid or isoleucine had no effect on the shedding, nor was the shedding affected when Lys-174 was replaced by glutamine or threonine. Replacement of Val-173 with aspartic acid, glycine, or arginine did seem to decrease the shedding somewhat, although the decrease was only partial (Fig. 7). In contrast, replacement of Val-173 with proline resulted in a marked decrease in the shedding. The shedding was also found to be strongly suppressed by replacement of Lys-174 or Gly-175 with proline. It was hardly affected, however, by substitution of proline for Asn-172, Thr-176, or Glu-177 (Fig. 8).

DISCUSSION

The knowledge acquired in this study, although gained solely by examining how modifications of the p55-R structure affect its shedding, provides useful clues as to the mechanism of cleavage, its specificity, and its mode of activation.

In certain respects, the shedding appears to be a rather nonspecific process. It is independent of the sequence properties (and indeed of the very existence of the intracellular domain of the receptor) and shows no dependence on the structure of its transmembrane domain. It seems to be independent of the structure of the cytokine-binding region in the extracellular domain, as indicated by the effective shedding observed with a chimera in which the cytokine-binding region was derived from the EGF-R and the spacer from the p55-R. Even in the spacer region, where cleavage of the receptor appears to occur, it was possible to introduce rather extensive changes with little effect on the shedding.

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to the spacer, transmembrane, and intracellular domains of the EGF-R; *4*, a chimera without spacer (the cytokine-binding region of the TNF-R linked to the transmembrane and intracellular domains of the EGF-R); *5*, a chimera containing both the spacer of the hu-p55-TNF-R and part of the spacer of the EGF-R (the cytokine-binding and spacer regions of the TNF-R linked to the spacer, transmembrane, and intracellular domains of the EGF-R). *C*, chimeras whose cytokine-binding domain was derived from the EGF-R. *6*, cytoplasmically truncated human EGF-R; *7*, cytoplasmically truncated human EGF-R, part of whose spacer was replaced with the spacer of the hu-p55-TNF-R. Both constructs were transiently expressed in COS-7 cells. Shown is the pattern of metabolically labeled proteins immunoprecipitated with monoclonal anti EGF-R antibody (or, as a control (*cont*), with anti hu-p55-TNF-R monoclonal antibody) from the growth media of the PMA-treated and untreated transfected COS-7 and nontransfected cells. The intensities of the soluble EGF-R bands, as assessed by scanning, were (in relative units) 0.62 and 0.34 with construct 6 and 3.73 and 32.98 with construct 7 for the control and PMA-treated cultures, respectively. No such band could be discerned in the products of the nontransfected cells. Induction of receptor shedding by PMA (20 nM in *B* and *C*) and PV (100 μM in *B*) and assessment and presentation of the shedding were as described under "Experimental Procedures." The initial binding of ^{125}I-labeled TNF and ^{125}I-labeled EGF was 1,990 ± 150, 12,000 ± 380, 16,940 ± 980, 5,560 ± 310, and 15,790 ± 740 cpm/cell sample for constructs 1–5, respectively, when expressed in the A9 cells and 2810 ± 505, 4,790 ± 390, 18,300 ± 800, 4,900 ± 140, 136,640 ± 6,950, and 58,940 ± 4,980 cpm/cell sample for constructs *1–4*, *6*, and *7*, respectively, when expressed in the COS-7 cells. T.M., transmembrane domain.

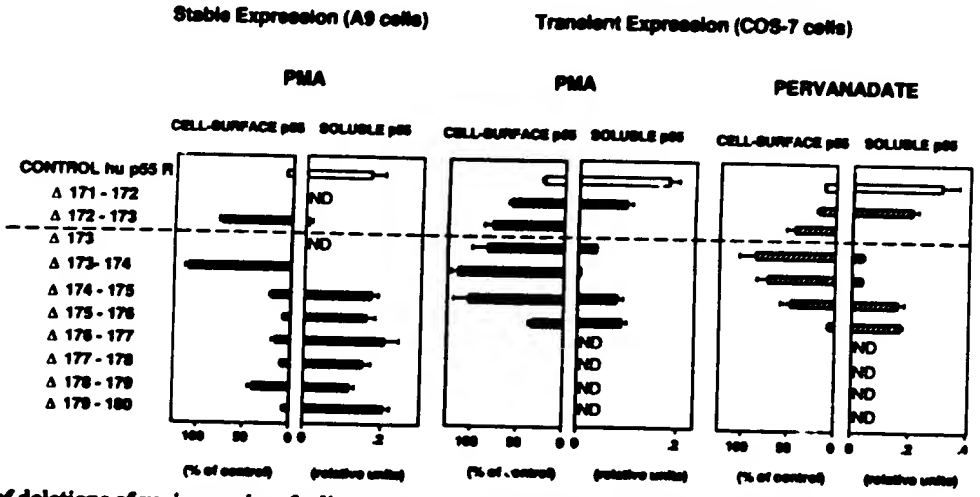

FIG. 5. **Effects of deletions of various pairs of adjacent amino acid residues in the hu-p55-TNF-R spacer on its shedding (assessed after treatment for 1 h with PMA (20 nM) or PV (100 μM)).** The putative major site of cleavage of the receptor (between residues 172 and 173) is indicated here, as well as in Figs. 6–8, by a *broken line*. *ND*, not determined.

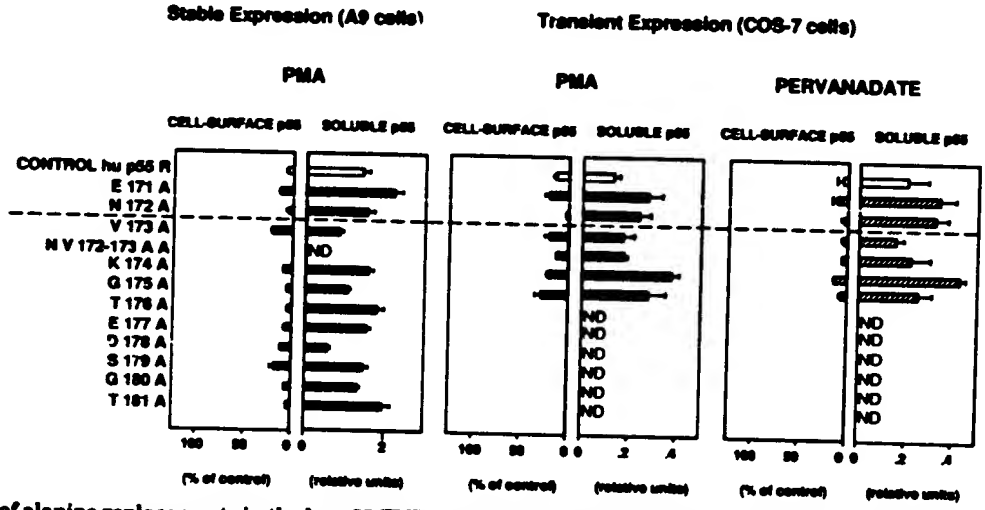

FIG. 6. **Effects of alanine replacements in the hu-p55-TNF-R spacer on its shedding (assessed after treatment for 1 h with PMA (20 nM) or PV (100 μM)).** *ND*, not determined.

The mechanism of shedding is, nevertheless, restricted by certain parameters of specificity. This is seen in the relative resistance to the shedding displayed by the cytoplasmically truncated EGF-R. Although we could discern some formation of a soluble form of this receptor, the fact that PMA treatment did not decrease the expression of the cell-surface EGF-R suggests that shedding of the EGF-R, even if it accounts for formation of this soluble form, occurs with much lower efficacy than shedding of the p55-R. This notion is further supported by the pronounced increase in PMA-induced shedding of the EGF-R when introducing the spacer of the p55-R to it and by the marked decrease in shedding efficacy of the p55-R when its spacer region was replaced by an EGF-R spacer sequence. The specificity of the shedding mechanism is further indicated by the fact that certain, rather limited, changes in the spacer region could, quite strongly, interfere with this mechanism. Two kinds of changes had such an effect: (i) deletion of Val-173 with either of its adjacent residues and (ii) replacement of residue 173, 174, or 175 with proline.

These data suggest that certain structural characteristics of the short spacer region in the receptor are essential and sufficient for its recognition by the protease involved in the shedding. More detailed analysis of these structural characteristics is needed for delineation of their distinctive features. Two such features have already emerged: (i) Val-173, located just downstream from the putative major site of cleavage of the receptor, seems to play an important role in the shedding, as indicated by the decrease in shedding efficacy when this residue is deleted or replaced by aspartic acid, glycine, or arginine. An important role of Val-173 in the shedding was also suggested in another study of this mechanism (44). The functional importance of Val-173 might underlie the conservation of this residue, as well as of Asn-172 in the human, rat, and mouse receptors (45, 46). (ii) Shedding seems to be strongly dependent on the conformation of the protein at the spacer region, particularly at the region of the presumptive cleavage site. Apart from its partial dependence on the identity of the residue at position 173, the shedding shows little dependence on the nature of the amino acid side chains in this region. The only changes found almost to eliminate the shedding were replacement of the residue at positions 173, 174, or 175 with proline. One possible explanation for the marked decrease in shedding when Val-173 is replaced by proline is that this change makes the peptide bond between residue 173 and Asn-172 resistant to cleavage; most proteases are unable to process the *X*-Pro bond (47). However, the fact that replacement of residue 174 or 175 with proline had the same inhibitory effect on the shedding suggests that there are some additional cause(s) of this proline replacement effect.

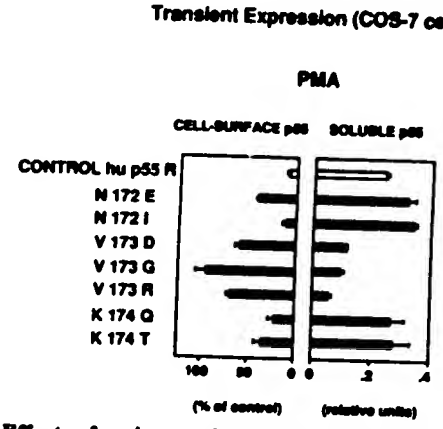

FIG. 7. **Effects of various amino acid replacements at positions 172, 173, and 174 in the hu-p55-TNF-R spacer on its shedding (assessed after treatment for 1 h with PMA (20 nM)).**

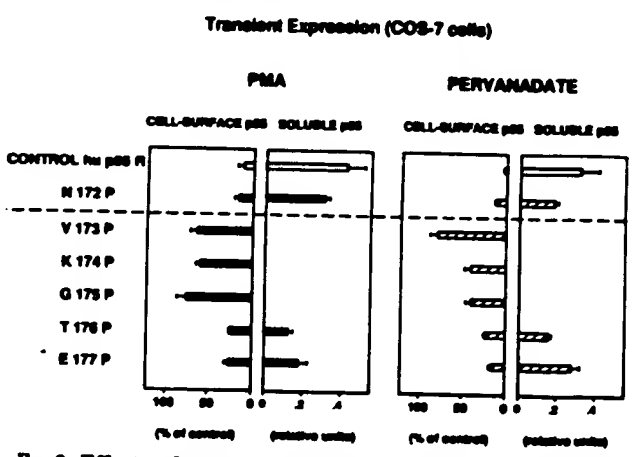

FIG. 8. **Effects of proline replacements in the hu-p55-TNF-R spacer on its shedding (assessed after treatment for 1 h with PMA (20 nM) or PV (100 μM)).**

It should be noted that replacement of Lys-174 or Gly-175 with other amino acids had no effect on the shedding, nor was shedding prevented by the deletion of either of these residues. Prevention of the shedding may well be due to distortion of the secondary structure of the protein in this region as a result of the steric constraints imposed by introduction of the proline side chain into the protein (47). Also, the strong decrease in shedding efficacy caused by deletion of 3 or 5 amino acids downstream from residue 174 may be due to distortion of the conformation of the spacer region. Alternatively, it could be due to a decreased accessibility of the space between the membrane and the rigid cytokine-binding region, resulting in a less efficient interaction of the protease with the p55-R.

The fact that cleavage of the p55-R is only slightly affected by the identity of the amino acid residues in the spacer region, even though being strongly dependent on its conformation, may reflect an involvement of multiple proteases with different sequence specificities but similar dependence on the conformation of the protein. It is equally possible, however, that the shedding is caused by a single protease that shows little sequence specificity yet strong dependence on the conformation of the protein at the cleavage site.

The nature of the substrate specificity observed in the shedding of the p55-R is reminiscent of the mode of function of the signal-peptide proteases (48). The dependence of these enzymes on the sequence of the cleavage site is not strict, and they can cut at various positions within a region sensitive to cleavage. Their activity appears, however, to be critically dependent on the secondary or tertiary structure of their substrate proteins. Whether this similarity reflects common mechanistic principles, and perhaps even an involvement of related proteases, in these two membrane-proximal cleavage processes remains to be determined. More detailed information on the substrate specificity in the shedding of the p55-R will be of help in the identification and isolation of the protease(s) involved in this process.

In principle, activation of the cleavage mechanism might occur as a consequence of an induced change in the receptor itself, which makes it more vulnerable to proteolysis or more accessible to the protease. Alternatively, it might be due to changes that affect the activity or localization of the protease. The observed effects of mutations in the p55-R on its shedding appear to support the second possibility. As previously noted, the fact that cytoplasmic truncation does not affect the shedding of the receptor seems to exclude involvement of the receptor in the intracellular events that trigger the shedding. In particular, it negates the possibility that the effect of PMA or PV on the shedding involves phosphorylation of the intracellular domain by a PMA-activated protein kinase C or by tyrosine kinases (19). Further evidence against an involvement of induced changes in the receptor itself in the triggering of its shedding comes from the fact that the shedding and its activation occur even when the transmembrane domain is replaced by the corresponding part of a receptor that is poorly shed. Use of the cytoplasmically truncated EGF-R in this analysis is particularly informative since it is evident that this receptor is not taken up into the cell in response to PMA. The fact that PMA-induced shedding of the p55-R was not prevented by replacement of its transmembrane and intracellular domains with th corresponding regions of a receptor that does not exhibit a PMA-induced uptake implies that the shedding is not a consequence of induced endocytosis. In an indirect way, this suggests that the shedding does not occur intracellularly but on the cell surface. This is supported by our observation that ammonium chloride and chloroquine, potent inhibitors of proteolytic processes occurring within intracellular acid compartments (49), have no effect on the induced shedding of the p55-R.

Shedding is an effective means of modulation of cell-surface protein expression. It can lead, rather rapidly, to the almost complete elimination of the cell-surface form of the p55-R and to the formation of significant amounts of its soluble form. In the absence of substrate specificity, operation of this mechanism would cause cells to be largely denuded of their cell-surface proteins, which would obviously have a devastating effect on the well being of the organism. The mechanism of shedding of the p55-R seems to be ubiquitously expressed. This is indicated by the observed similarity in effects of mutations on the shedding in the mouse A9 and monkey COS-7 cells. Only part of the mutants examined in this study were tested both in the A9 and in the COS-7 cell lines. However, the general patterns of mutant effects in the two lines strongly suggest that their shedding of the p55-R results from the action of the same or very similar protease(s). Various other cells have been shown to shed their p55-R in response to PMA, and probably also in these cells the same or a very similar mechanism is involved (15, 16, 19). It appears that a wide range of activating stimuli converge in this particular cleavage mechanism. Changes in intracellular serine/threonine phosphorylation as well as in tyrosine phosphorylation can both lead to its activation, as suggested by the similar substrate requirements for PMA- and PV-induced cleavage. A study showing that shedding of the p55-R can be triggered by okadaic acid suggested that serine protein kinases other than protein kinase C may also trigger this process (50). Being responsive to different protein-phos-

phosphorylation mechanisms, the shedding is liable to be triggered by a wide range of biological stimuli.

It is quite possible that there are other proteins that are shed by the same mechanism as that affecting the p55-R. However, as exemplified in this study by the data on the shedding of the EGF-R, there are clearly also proteins that are resistant to it. Moreover, preliminary information on the structural requirements for the shedding of other cell-surface proteins indicates that shedding can also occur by other mechanisms. Shedding of the cell-surface-expressed TGF-α, for example, does seem to depend on some function(s) of the intracellular domain of this protein. It is prevented when the 2 C-terminal residues of TGF-α (both of them valines) are deleted or replaced (51). Shedding of the p75-TNF-R by granulocytes involves, in part, its cleavage by elastase, a soluble protease that recognizes a rather well defined sequence motif (52). In contrast, shedding of the p55-R seems, even in the granulocytes, to occur solely as a result of the activity of some cell-bound protease(s) (52), and, as indicated by the data of the present study, these protease(s) do not seem to recognize a single distinct sequence motif. Involvement of multiple proteolytic systems in cell-surface protein shedding is also indicated by the differential sensitivities of different shedding events to various protease inhibitors (53).

Occurrence of different and independent mechanisms for the shedding of different cell-surface proteins seems to make sense from a physiological standpoint in view of the different physiological functions served by this process. A number of soluble receptors can, like the soluble TNF-Rs, compete with the cell-surface forms for their agonists and thus inhibit its function. Others, like the soluble interleukin-6 receptor, are able to substitute for the cell-surface receptors (54). There are also receptors, like CD23 (the low affinity IgE receptor), which, once shed, function as agonists (55). The soluble forms of many cell-surface proteins may have no function at all but merely constitute a side product of the normal turnover of their cell-surface forms or of their down-regulation mechanisms. Further study of the structural requirements for shedding of the p55-R will allow us to determine which other cell-surface proteins are cleaved in the same way, thus providing more comprehensive understanding of the physiological significance of this cleavage mechanism.

Acknowledgments—We thank A. Dibeman and S. Maizel for careful handling of the cultured cells. We also thank Dr. H. Hauser for advice and for providing the pMPSVEH vector, Dr. Irit Alkalay for providing the pEXV1 vector, Drs. G. Merlino and I. Pastan for the cDNA of the human EGF-R, Dr. D. Givol for the cDNA of the mouse EGF-R, Dr. A. Ulrich for the monoclonal anti-EGF-R antibodies, and Dr. Y. Shechter for advice.

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